Development of new epigenetic-based biomarkers for renal cell tumors with clinical application

Definição de novos biomarcadores com potencial de aplicação clínica com base no perfil epigenético dos tumores renais

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STATEMENT DECLARAÇÃO

Ao abrigo do Art.º 8º do Decreto-Lei n.º 388/70, fazem parte integrante desta dissertação os seguintes manuscritos publicados ou submetidos para publicação:

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Em cumprimento do disposto no referido Decreto-Lei, a candidata declara que participou activamente na definição dos objectivos, bem como na recolha e estudo do material incluído nos trabalhos 2 e 3, bem como na análise e interpretação de resultados, e redacção do artigo, em colaboração com os restantes co-autores. Participou também activamente na definição de objectivos, recolha e estudo do

material incluído nos trabalhos 4 e 5 (selecção de casos, extração de RNA, síntese de cDNA, avaliação da expressão de genes por PCR quantitativo em tempo real, avaliação imunohistoquímica), bem como na análise e interpretação de resultados e redação do artigo, em estreita colaboração com a outra primeira autora *ex aequo* dos referidos artigos, no âmbito das teses de Mestrado das referidas co-autoras.

Colaborou ainda activamente na redação do trabalho 1, e na seleção de casos e recolha de informação clínica dos casos usados no trabalho 6, no âmbito da tese de Mestrado do primeiro autor.

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To the loving memory of

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(15/06/1933 – 12/06/1998)

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ABSTRACT

Renal cell tumors encompass a heterogeneous group of neoplasms, with distinct clinical, morphological, genetic and epigenetic features. Despite molecular similarities, revealed by large multilevel comparative studies, these molecular differences might portend distinct prognosis and dissimilar response to targeted therapies. Both genetic and epigenetic events may disrupt the main cellular pathways altered in clear cell renal cell carcinoma, papillary renal cell carcinoma, chromophobe renal cell carcinoma and oncocytoma. Despite epigenetic alterations – as gene promoter hypermethylation, histone oncomodification and altered miRNAs expression – have been described as promising biomarkers for detection and discrimination among different renal cell tumors as well as prognostication, validation in clinical series is still lacking, precluding its translation to the clinical setting.

Thus, the main aim of this Thesis was to assess the clinical usefulness of epigenetic-based biomarkers (mainly promoter methylation and histone modifying enzymes expression) in renal cell tumors, especially their diagnostic and prognostic value.

Concerning methylation profile of renal cell tumors, a panel comprising the promoter methylation levels of OXR1 and MST1R was found to be a highly sensitive and specific diagnostic biomarker for renal cell tumors (98% sensitivity, 100% specificity) and for clear cell renal cell carcinoma (90% sensitivity, 98% specificity). Moreover, MST1R promoter methylation was associated with transcription regulation in renal cell tumors, and MST1R expression was associated with prognosis.

Regarding histone modifying enzymes, *SMYD2*, *SETD3*, *NO66*, *SETDB2* and *RIOX2* (histone demethylases or methyltransferases) were identified as differentially expressed in renal cell tumors and validated in a cohort of 160-cases as well as in TCGA database. Moreover, low SMYD2, SETD3 and NO66 expression levels were associated with shorter disease-specific survival, and low SMYD2, SETD3, NO66 and SETDB2 with shorter disease-free survival (in multivariable analysis). *RIOX2* expression level was also associated with the development of metastasis during follow-up.

In conclusion, epigenetic alterations constitute promising diagnostic biomarkers, as promoter methylation and microRNAs, and promising prognostic biomarkers, as histone modifying enzymes, for clinical management of renal cell tumors. Additional assessment in large multicenter trials is needed to support clinical implementation of these biomarkers.

RESUMO

Os tumores de células renais compreendem um grupo heterogéneo de neoplasias, com características clínicas, morfológicas, genéticas e epigenéticas distintas. Apesar das semelhanças a nível molecular, reveladas por estudos comparativos, as diferenças moleculares podem condicionar diferentes prognósticos e resposta diferente a terapias dirigidas. Tanto mecanismos genéticos como processos epigenéticos podem alterar as principais vias de sinalização celular desreguladas nos carcinomas de célula renais de tipo célula clara, papilar, cromófobas e em oncocitoma. Apesar de as alterações epigenéticas – como a hipermetilação do promotor de genes, oncomodificação de histonas e expressão alterada de microRNAs – terem sido descritas como biomarcadores promissores para a deteção, discriminação e avaliação de prognóstico em tumores de células renais, não existe validação extensa em séries clínicas, impedindo a sua utilização em contexto clínico.

Assim, o principal objetivo desta Tese foi determinar a utilidade clínica de biomarcadores baseados em alterações epigenéticas (em especial hipermetilação do promotor e alteração da expressão de enzimas modificadoras de histonas) em tumores de células renais, nomeadamente o seu valor como biomarcadores de diagnóstico e prognóstico.

Em relação ao perfil de metilação dos tumores de células renais, um painel constituído pelo nível de metilação do promotor de *OXR1* e *MST1R* mostrou ser um biomarcador de diagnóstico com elevada sensibilidade e especificidade na identificação de tumor de células renais (sensibilidade de 98% e especificidade de 100%) e de carcinoma de

células renais de tipo célula clara (sensibilidade de 90% e especificidade de 98%). Adicionalmente, o padrão de metilação do promotor do gene *MST1R* foi associado à regulação da transcrição em tumores de células renais, e a expressão de *MST1R* foi associada ao prognóstico.

Relativamente às enzimas modificadoras de histonas, o nível de expressão de *SMYD2*, *SETD3*, *NO66*, *SETDB2* e *RIOX2* (histonas desmetilases ou metiltransferases) foi distinto em tumores de células renais diferentes, resultado este validado numa série de 160 tumores de células renais e nos casos da base de dados do TCGA. Um nível baixo de expressão de *SMYD2*, *SETD3* e *NO66* foi associado a uma menor sobrevivência específica por doença, e nível baixo de expressão de *SMYD2*, *SETD3*, *NO66* e *SETDB2* a uma menor sobrevivência livre de doença (em análise multivariável). O nível de expressão de *RIOX2* também foi associado ao desenvolvimento de metastização durante o follow-up.

Em conclusão, as alterações epigenéticas constituem-se como promissores biomarcadores de diagnóstico, como a metilação do promotor e a expressão de microRNAs, e como promissores biomarcadores de prognóstico, como a expressão de enzimas modificadoras de histonas, com potencial de aplicação clínica em tumores de células renais. Será necessária avaliação adicional em grandes ensaios multicêntricos para suportar a implementação clínica destes biomarcadores.

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ABBREVIATIONS LIST

AKT: AKT serine/threonine kinase
ALK: ALK receptor tyrosine kinase
ARID: AT-rich interaction domain
ATM: ATM serine/threonine kinase
AUC: area under the curve

BAP1: BRCA1 associated protein 1

CCND1: cyclin D
ccRCC: clear cell renal cell carcinoma
CDH1: cadherin 1
CDKN2A: cyclin dependent kinase inhibitor 2A
chRCC: chromophobe renal cell carcinoma
CI: confidence interval
CNV: copy number variation
CTNNB1: catenin beta 1

DFS: disease-free survival
DICER-1: dicer 1, ribonuclease III
DNA: deoxyribonucleic acid
DNMT: DNA methyltransferases
DSS: disease-specific survival

EMT: epithelial to mesenchymal transition

H2A: histone 2A
H2B: histone 2B
H3: histone 3
H3K27ac: H3 lysine 27 acetylation
H3K27me3: H3 lysine 27 trimethylation
H3K36me3: H3 lysine 36 trimethylation
H3K4me1/me2: histone 3 lysine 4 methylation/dimethylation
H3K4me2/me3: H3 lysine 4 di/trimethylation
H3K79me2: H3 lysine 79 dimethylation
H3K9me2/me3: H3 lysine 9 di/trimethylation
H4: histone 4
HAT: histone acetyltransferse
HDAC: histone deacetylase

HDM: histone demethylase
HIF: hypoxia inducible factor
HMT: histone methyltransferase
HOXA9: homeobox A9
HR: hazard ratio
HRAS: HRas proto-oncogene, GTPase

ISUP: International Society of Urologic Pathology

KDM: lysine demethylase
KDM5C / JARID1C: lysine demethylase 5C
KDM6A / UTX: lysine demethylase 6A

LAD: lamina-associated domains
LOCK: large organized chromatin K9 modifications
LRES: long-range epigenetic silencing domains

MDR1/ABCB1: ATP binding cassette subfamily B member 1
MEK/MAP2K7: mitogen-activated protein kinase kinase 7
MET: MET proto-oncogene, receptor tyrosine kinase
miRNA: microRNA
MiT: MET proto-oncogene, receptor tyrosine kinase
MST1R: macrophage stimulating 1 receptor
MT-ND: mitochondrially encodedNADH
mTOR: mechanistic target of rapamycin

NO66/RIOX1: ribosomal oxygenase 1
NPV: negative predictive value

OXR1: oxidation resistance gene 1

PBRM1: polybromo 1
PCR: polymerase chain reaction
PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase
PPV: positive predictive value
pRCC: papillary renal cell carcinoma
PTEN: phosphatase and tensin homolog

QMSP: quantitative methylation specific polymerase chain reaction

RASSF1A: Ras association domain family member 1

RCC: Renal Cell Carcinoma
RCT: Renal Cell Tumor
RIOX2: ribosomal oxygenase 2
RNA: ribonucleic acid
RNT: renal normal tissue
ROBO1: roundabout guidance receptor 1
ROC: receiver operating characteristic

SE: sensitivity
SETD2: SET domain containing 2
SETD3: SET domain containing 3
SETDB2: SET domain bifurcated 2
SFRP: secreted frizzled-related protein
SIRT1: sirtuin 1
SLIT2: slit guidance ligand 2
SMARC: switching defective/sucrose nonfermenting (SWI/SNF) related, matrix associated, actin dependent regulators of chromatin
SMYD2: SET and MYND domain containing 2
SP: specificity
SWI/SNF complex: switching defective/sucrose nonfermenting

TAD: topologically associating domains
TCEB/ELOC: elongin C
TCGA: The Cancer Genome Atlas
TERT: telomerase reverse transcriptase
TET: tet methylcytosine dioxygenase
TGFB: transforming growth factor beta
TP53: tumor protein p53
TRBP: TARBP2, RISC loading complex RNA binding subunit
TSC: tuberous sclerosis

VEGF: vascular endothelial growth factor
VHL: von Hippel-Lindau tumor suppressor

WHO: World Health Organization

THESIS OUTLINE

This thesis is organized in six chapters and one appendix.

Chapter 1 contains an introduction to the thesis theme, including a review on renal cell tumor classification, main epigenetic mechanisms, and the most frequent and consistently described alterations in renal cell tumors, both genetic and epigenetic. The second part of this chapter focuses on the potential clinical application of the epigenetic alterations in renal cell tumors.

Chapter 2 defines the aims of this thesis.

Chapter 3 enumerates the experimental procedures performed by the candidate, since detailed description is depicted in the methods section of each study.

Chapter 4 is composed of two studies on DNA methylation of renal cell tumors. The first describes the diagnostic significance of a panel including the promoter methylation level of three genes. The second explores the prognostic and functional role of *MST1R* promoter methylation.

Chapter 5 is composed of two studies focusing on histone modifying enzymes. These two studies were performed in close collaboration with two distinct Master students (acknowledged as joint first authors of the respective articles) as part of their Master

Degree project. For the purpose of these studies, I selected cases and I was actively involved in performing RNA extraction from fresh frozen tissue, cDNA synthesis, quantitative real time PCR, data analysis and interpretation, and manuscript writing. The first study identified histone methyltransferases and demethylases differentially expressed among renal cell tumors and assessed the role of three of them as diagnostic and prognostic biomarkers. The second study assessed the prognostic value of other two histone modifying enzymes as prognostic and disease progression biomarkers.

Chapter 6 contains the general discussion of the main results of this thesis, as well as the main conclusion and future perspectives.

The **appendix** contains a study on microRNAs as diagnostic biomarkers in renal cell tumors, performed by a Master Degree student as part of his Master project. I collaborated by selecting cases, retrieving relevant information from clinical charts and participating in data interpretation and manuscript review.

CHAPTER 1

GENERAL INTRODUCTION

1.1. CHAPTER OVERVIEW

This chapter includes a review paper published in international peer reviewed journal:

- *Henrique R, **Luís AS**, Jerónimo C. The epigenetics of renal cell tumors: from biology to biomarkers. Front Genet, 2012; 3:94.*

Renal cell tumors encompass a heterogeneous group of neoplasms, with distinct clinical, morphological, genetic and epigenetic features [1, 2]. The relevance of molecular alterations specific for each subtype was reflected by the relatively early inclusion of cytogenetic features in renal cell tumor classification [3, 4]. Despite some molecular similarities, underscored by large multilevel comparative studies [5], these molecular differences might portend distinct prognosis and dismal response to targeted therapies [1, 6].

Over the last years, integrative studies revealed the molecular features of ccRCC [7], pRCC [8], chRCC [9] and oncocytoma [10], revealing the main cell pathways altered in each subtype, mostly by genetic events. These pathways were also frequently altered by epigenetic alterations [2], and thus it was felt that a brief review of the main genetic and epigenetic processes involved in each histotype tumorigenesis, in the first part of this chapter, was essential to highlight the relevance of epigenetic alterations in renal cell tumors.

Moreover, these epigenetic alterations might be useful in renal cell tumor diagnosis and prognostication, which would be promising new tools for renal cell tumor

management, and are reviewed in the second part of this chapter, in the published review [11].

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1.2. INTERMINGLING MORPHOLOGICAL AND MOLECULAR FEATURES – INSIGHTS ON RENAL CELL TUMOUR CLASSIFICATION AND PATHOGENESIS

1.2.1. KIDNEY CANCER

Kidney cancer incidence is increasing worldwide, with 338000 estimated new cases and 143000 estimated deaths in 2012. Incidence and mortality rates are higher in men and in more developed regions. Although incidence rate has been increasing in the last years, more in men than women, the mortality rate has been stable and even decreasing in most high resource countries (Northern and Western Europe, USA, Australia) since the 90s, more in women than men. The ratio between incidence and mortality is highest in Northern America, indicating a higher survival than in Africa, where this ratio is lowest [1]. The increasing incidence rate has been largely attributed to the growing number of incidental small renal tumours diagnosed due to widespread use of imaging techniques, mostly in high resource countries, in addition to ageing, obesity and smoking, which are known risk factors for the development of kidney cancer. The stabilization or even slightly decrease trend in mortality rate might be related to efficient treatment by surgery in localized cancer, as well as interferon and targeted therapies used mainly in high resource countries. The rising number of small neoplasms incidentally diagnosed, mostly in high resource countries, could also contribute to this mortality trend, not being clear yet if the decreasing mortality corresponds to a survival gain, as the prognosis of these small tumours is generally good and most are cured by partial nephrectomy. A higher proportion of this small tumours diagnosed in high resource countries, in addition to more access to targeted therapies, could contribute to the differences in the ratio between incidence and mortality observed in high resource vs. low resource countries [1].

The vast majority (90%) of kidney cancers are renal cell tumours (RCT), arising from renal parenchyma. The remaining are mostly urothelial carcinomas, arising from renal

pelvis. Most RCT are sporadic; however some familiar forms are well documented, including von Hippel-Lindau syndrome, hereditary papillary renal cell carcinoma, familial leiomyomatosis and renal cell carcinoma syndrome and Birt-Hogg-Dubé syndrome [2]. RCTs are a heterogeneous group and include benign tumours, such as oncocytoma (5-9%); and malignant entities, called renal cell carcinomas (RCC), which encompass different tumour types with dissimilar prognosis [3].

The most frequent RCC is clear cell renal cell carcinoma (ccRCC) (70-75%), which also have the worst prognosis among the most prevalent subtypes, followed by papillary renal cell carcinoma (pRCC) (15-20%), with slightly better prognosis than ccRCC. Chromophobe renal cell carcinoma (chRCC) is less frequent (5-7%) and presents a better prognosis than ccRCC and pRCC. For each subtype, the prognosis is worst in tumours with metastasis, which are more frequent in ccRCC and pRCC, and rare in chRCC [3]. Approximately 20 to 30% of all patients present metastases at diagnosis, and approximately 20% develop metastases after nephrectomy [2].

1.2.2. SPORADIC RENAL CELL TUMOURS

1.2.2.1 Conceptual and temporal framework of renal cell tumours classification

The first kidney tumour classifications were based on macroscopic appearance (Koning, in 1826) and on macroscopic and clinical feature (Rayer, in 1841). Later, in the beginning of the 20th century, some classifications based on the histological features were proposed, mostly descriptive, comprising a variable number of entities and mirroring the then accepted idea that renal tumours arose from adrenal remnants, originating the term hypernephroma [4, 5].

Although it was initially thought that renal tumours derived from renal tubular epithelial cells, according to Robin (in 1855) and Waldeyer (in 1867) observations, after Grawitz description (in 1884) of small yellow subcapsular renal tumours composed by clear cells as being originated from intrarenal adrenal rests (whereas small papillary tumours originated in renal tubules) the concept of ectopic adrenal rest origin was generalized for all renal tumours. The controversy remained until 1959, when Oberling observed that clear cells from renal tumours presented ultrastructural features similar to renal tubular epithelial cells [5]. Latter classifications further emphasized this renal origin, by designating these neoplasms as “renal cell tumours”.

Noteworthy, these descriptive classifications included benign and malignant entities, despite their distinction was neither straightforward nor uncontroversial, with size being the most widespread criteria to distinguish them, after Bell reported a low metastasization rate for tumours smaller than 3cm in 1928 [4, 6]. Despite reference to papillary tumours and to clear and granular cells was common in these classifications, the first WHO classification of renal cell tumours in 1981 did not include these morphotypes, but acknowledged the existence of large morphological variety in renal cell tumours.

A seminal paper published in 1986 and latter known as “Mainz classification” set the basis for subsequent classification schemes [6]. Tumours were characterized considering cytology and architecture, the main subtypes being clear cell, chromophobe, chromophilic (generally with papillary architecture) and oncocytoma. This was the first study to recognize that granular cells could appear in all the described subtypes, not being an independent subtype as previously thought, and that sarcomatoid change is a manifestation of dedifferentiation of the remaining cancer

subtypes. It also included an “unclassifiable renal cell tumour” category, to prevent unclear cases to be classified as a defined tumour morphotype [6]. Subsequent studies expanded the number of identified subtypes and compared these neoplasms to renal tubules epithelial cells, concluding that clear cell and chromophilic cell (papillary) variants originated from proximal tubules, chromophobe cell from intercalated cells of the cortical collecting duct, oncocytoma from cortical collecting duct and Bellini duct variant from principal cells of the medullary collecting duct [7]. This pathomorphological classification was also supported by independent cytogenetical and molecular studies [8-10] that reported specific cytogenetic alterations for each morphological subtype, as depicted bellow.

Thus, the need for integration of these molecular data and morphological criteria in a single classification lead to the Heidelberg/Rochester classification (1997), the first using simultaneously morphological [11] and cytogenetic [12] data to design a new classification scheme for renal cell tumours, including benign tumours as papillary adenoma, oncocytoma and metanephric adenoma, as well as malignant entities as conventional (clear cell), papillary and chromophobe renal cell carcinoma, collecting duct carcinoma, medullary carcinoma and renal cell carcinoma unclassified. One remarkable feature of this classification was the strict morphologic criteria used to define each morphotype, the use of genetic alterations to classify some dubious cases, and the definition of an unclassified category of tumours not meeting the strict morphological features and devoided of the typical genetic features of each subtype [11, 12].

This allowed for a more precise definition and knowledge of the prognosis of the early defined and more frequent histotypes, and contributed for the identification,

characterization (clinical, morphological, genetic) and subsequent description of novel RCC subtypes, four of which were included in the 2004 WHO classification [13]: multilocular clear cell renal cell carcinoma, Xp11 translocation carcinomas, renal cell carcinoma associated with neuroblastoma and mucinous tubular and spindle cell carcinoma.

Accumulating evidence on each subtype common and infrequent morphology, prognosis, genetic and epigenetic alterations, in addition to the description of several novel renal tumours, lead the International Society of Urologic Pathology (ISUP) to promote a consensus conference in Vancouver in 2012, to update the classification of renal cell tumours [14]. Some entities were redefined, as multilocular cystic clear cell renal cell neoplasm of low malignant potential and MiT family translocation renal cell carcinoma, that includes Xp11 translocation renal cell carcinoma and t(6;11) renal cell carcinoma; and five novel entities were recognized, including tubulocystic renal cell carcinoma, acquired cystic disease-associated renal cell carcinoma, clear cell (tubulo) papillary renal cell carcinoma and hereditary leiomyomatosis renal cell carcinoma syndrome-associated renal cell carcinoma, as well as three provisional entities. The Vancouver classification set the basis for the 2016 WHO classification [3], which includes the new entities recognized by the Vancouver classification, as well as succinate dehydrogenase-deficient renal cell carcinoma; and also recognizes four provisional entities: oncocytic renal cell carcinoma occurring after neuroblastoma (removed from 2004 WHO and Vancouver classification), thyroid-like follicular renal cell carcinoma, ALK rearrangement-associated renal cell carcinoma and renal cell carcinoma with (angio)leiomyomatous stroma.

It should be noted that each subsequent renal cell tumour classification system further describes more precise clinicopathological and molecular features of well established entities, redefines some entities based on newly available data, and incorporates new entities when their morphologic and genetic features are consistently reported as different from the recognized renal cell tumours. So, they are dynamic frameworks for both precise diagnostic and further clinical, genetic and epigenetic research, since a precise definition of tumour entities is the basis of case selection for further studies and allows for the identification of new diagnostic, prognostic and predictive markers, specific for each tumour type and potentially more useful for clinical practice.

Several of such studies have been published, including mutation analysis, gene expression analysis, methylation profiling and microRNA analysis, which are broadening our knowledge about renal carcinogenesis, not only the role of genetic alterations but especially the role of epigenetic alterations.

1.2.2.2 Role of epigenetic alterations in renal tumorigenesis

1.2.2.2.1 General concepts of epigenetic deregulation and carcinogenesis

Epigenetics, first mentioned by Waddington in 1942, was subsequently defined as heritable cellular information, for instance gene expression patterns, not due to changes in the DNA sequence [15, 16]. Epigenetic alterations comprise DNA modifications, histone modifications and microRNA regulation, which might change the gene expression pattern of cells. These alterations are closely interrelated and, unlike genetic changes, might be reversible, upon action of specific enzymes [17]. Epigenetic mechanisms are critical to embryologic development, and some epigenetic alterations, as histone modifications, are involved in kidney development [18].

Epigenetic alterations were first recognized in cancer in 1983 [15, 16]. In parallel with the cytogenetic alterations, integrated in renal cell tumour classification since 1997 [11, 12], epigenetic alterations were early recognized as frequent in these tumours [19-21]. It is now widely accepted that epigenetic mechanisms are pivotal to renal cell oncogenesis, and the amount and variety of epigenetic alterations described in RCT is still increasing, some of them carrying diagnostic or prognostic relevance [22].

1.2.2.2.2. DNA modifications

The most well known DNA modification is cytosine methylation in CpG dinucleotides, occurring at the 5^{th} carbon of the cytosine located 5' of a guanosine (C5-methylcytosine; 5mC), mediated by DNA methyltransferases (DNMT). DNA regions with high proportion of CpG dinucleotides have been called CpG islands, and it was proved by 1980 that DNA methylation at CpG islands located at a gene promoter was associated with gene repression [15, 17]. Other more recently described DNA modifications are N3-methylcytosine (3mC), catalysed chemically; N6-methyladenine (6mA), without known mechanism of modification; and C5-hydroxymethylcytosine (5hmC), C5-formylcytosine (5fC) and C5-carboxylcytosine (5caC), resulting from active demethylation of 5mC by TET enzymes and subsequent oxidation [23].

The first epigenetic alteration described in cancer cells (1983) was global loss of DNA methylation, i.e., hypomethylation [24, 25], which may lead to the activation of gene expression, including that of oncogenes as *HRAS* or genes associated with drug resistance as *MDR1,* as well as to chromosomal instability [16], predisposing to abnormal recombination and facilitating deletions, translocations and chromosomal rearrangements [26]. Later on, in the 1990s, hypermethylation of tumour suppressor

genes (TSG) promoters, with associated gene inactivation, was consistently described for several tumours [16], including VHL promoter hypermethylation in renal carcinoma, in 1994 [21]. Besides CpG islands in gene promoters, methylation of other DNA regions was associated with gene expression regulation, as CpG shores (low density CpG regions near CpG islands) [27] and gene enhancers. Indeed, enhancer altered DNA methylation was proposed to more closely relate with gene expression changes than promoter methylation itself, which might be in part due to altered transcription factor binding [28]. Additionally, DNA methyltransferases were found to be commonly overexpressed in cancer [29].

Thus, DNA methylation has emerged as an important epigenetic modification in cancer: both global DNA hypomethylation and locus-specific promoter hypermethylation were reported as early events in tumorigenesis [26], and characteristic hypermethylation profiles of TSG genes were described for some cancer types [20]. Several genes have been reported as hypermethylated in RCC, first identified by a candidate-gene approach, and later by a functional epigenomic approach (comparing gene expression without and after treatment with demethylating drugs to identify genes re-expressed after treatment, which likely correspond to genes silenced by promoter methylation). Genomewide CpG methylation analysis platforms were then used to identify putative new TSG in RCC and to disclose differences in methylation patterns among RCT types (reviewed in [30]). For most reported genes, the frequency of hypermethylation was lower than 70%, and some genes identified in genomewide methylation platforms were not validated by more sensitive and specific techniques [30]. However, it was noted that hypermethylated genes in RCC were involved in some frequently dysregulated pathways (Tables 1-7), including WNT – β-

catenin, SLIT-2 – ROBO1, epithelial-to-mesenchymal transition (EMT) and metabolic pathways; as well as in cell cycle regulation, apoptosis and angiogenesis [22, 30].

1.2.2.2.3. Histone modifications

The DNA strand associates with proteins, named histones, to form chromatin. The nucleosome, considered the basic unit of chromatin organization, is formed by a histone octamer (two copies of H2A, H2B, H3 and H4) core, packaging 147 bp of DNA. Each histone has a N-terminal tail, and histone aminoacid residues might be chemically modified, the most frequent being acetylation, methylation or ubiquination of lysine residues, phosphorylation of serine residues and methylation of arginine residues. These modifications, forming the so-called “histone code”, are added and removed by specific enzymes, control gene expression, DNA replication and repair, and chromosome organization [15, 22, 23]. There are currently 12 chemical modifications and at least 130 potential modification sites at the tails of the 4 canonical and 30 histone variants [23]. The “histone code” implies that the combination of histone modifications pattern entail distinct biological outcomes, as gene expression or repression and chromatin remodeling, partially by recruiting downstream effector proteins or protein complexes [17]. For instance, gene expression is associated with histone H3 lysine acetylation and H3 lysine 4 di and trimethylation (H3K4me2 and H3K4me3) in the promoter; with H3 lysines 36 and 79 methylation (H3K36me3 and H3K79me2) in the gene body; and with H3 lysine 27 acetylation (H3K27ac) and lysine 4 methylation (H3K4me1 and H3K4me2) in enhancers. Conversely, gene repression is associated with H3 lysine 27 trimethylation (H3K27me3) and H3 lysine 9 methylation (H3K9me3), in the promoter, gene body (H3K9me2 and H3K9me3) and enhancer

(H3K9me2 and H3K9me3) [31]. Globally, histone acetylation is associated with gene expression, and histone methylation could be associated with gene expression or repression depending on the modified aminoacid. Besides these chromatin marks (specific chemical modifications), gene expression and chromatin structure is also related to chromatin features (several linked modifications and complex elements), as nucleosome occupancy, chromatin interactions and chromatin domains [topologically associating domains (TAD), lamina-associated domains (LAD), long-range epigenetic silencing domains (LRES), large organized chromatin K9 modifications (LOCK)] [23].

Several chromatin modifying enzymes have been described, the most extensively studied being histone acetyltransferases (HAT), deacetylases (HDAC), methyltransferases (HMT) and demethylases (HDM), involved in the establishment, maintenance or removal of covalent histone modifications, important for normal cell differentiation; as well as chromatin remodeling protein complexes, like *SWI/SNF* and *BAF*-associated complexes, recruited to condense or decondense chromatin, enabling gene silencing or activation, respectively [17, 22, 31].

In neoplastic cells, histone regulating mechanisms might be altered, not only covalent histone marks, but also non-covalent chromatin remodeling mechanisms as nucleosome positioning alterations and histone variants incorporation [17]. Indeed, cancer cells were found to present altered methylation patterns of H3K9 and H3K27 associated with gene repression, and different expression patterns of histone modifying enzymes from normal tissue, which might differ among tumour types and contribute to tumour initiation and progression [15, 32].

It is thought that the deregulation of these mechanisms is relevant for renal carcinogenesis, as mutations in chromatin modification and remodeling genes, like

PBRM1, *BAP1*, *SETD2*, *KDM5C* and *KDM6A* [33-35], which were found to be frequent in RCCs, only surpassed by VHL mutations (Table 2). These enzymes are involved in genomic integrity maintenance, cell-adhesion regulation, *HIF* signaling and cell cycle control [22].

It should be noted that some histone modifying enzymes can also target non-histone proteins, and their deregulation might affect cell phenotype not only by altering gene activity but also other cellular proteins [23]. Moreover, chromatin modifications and DNA methylation are functionally linked – DNMTs participate in multiprotein complexes with HDACs and HMTs to repress gene expression (Polycomb-mediated silencing), and some CpG islands display high affinity to transcription factors (as *CFP1*) that recruit activating HMTs and prevent DNA methylation [15, 17].

1.2.2.2.4. microRNAs

The first microRNA (miRNA) was described in 1993, and by 2001, the miRNA regulatory role was broadly, whereas altered miRNA expression in cancer was reported in 2005, and since then the number of altered miRNA and the complexity of miRNA deregulation networks in cancer cells has substantially increased [36-38]. miRNAs are a class of short (19-25bp) non-coding RNAs that bind to complementary sequences in mRNA, targeting them for degradation, and, thus, mediate post-transcriptional gene silencing. Most miRNA are encoded by specific miRNA loci, whereas approximately 30% originate from introns of protein coding genes. The primary miRNA (pri-miRNA) transcribed by RNA polymerase II are cleaved in the nuclei by a protein complex of DROSHA (a double-stranded RNase III enzyme) and its cofactor DGCR8 to a 60-70bp hairpin-shaped precursor miRNA (pre-miRNA), which is exported to the cytoplasm by

exportin 5 (XPO5). Pre-miRNA are then processed by DICER1 (a RNase III enzyme), which performs an asymmetrical cleavage of the dsRNA close to the terminal loop sequence, producing the mature miRNA duplex with 2-nucleotide 3' overhangs. DICER1 associates with TRBP, which binds to dsRNA, enhancing the fidelity of cleavage for a pre-miRNA subset and triggering the formation of iso-miRNAs (1 nucleotide longer than the regular miRNA), and physically bridges DICER1 to the Argonaute proteins. Mature miRNA, DICER1 and other specific proteins form the RNA-induced silencing complex (RISC). In RISC complex, an Argonaute protein binds one strand of the mature miRNA (guide strand) so that its 5' end (seed region, between nucleotides 2-8) is positioned for interaction with the 3' untranslated region (3'UTR) of target mRNA. RISC complex binding of complementary target mRNA results in faster mRNA degradation due to accelerated shortening of mRNA poly(A) tail [37, 39]. It is considered that the complementarity between miRNA seed region and target mRNA is crucial for post-transcriptional regulation: perfect complementarity leads to Ago-catalyzed cleavage of target mRNA, whereas imperfect complementarity leads to mRNA translation repression [38].

A single miRNA can target several mRNA, and distinct miRNA can target the same mRNA, creating complex networks. In these networks, as multiple genes can be simultaneously regulated by an individual miRNA, the fine-tuning by regulation distinct sub-networks is facilitated, and, when multiple target mRNA participate in the same signaling pathway, even modest inhibition of multiple targets can generate a stronger response than total inhibition of a single target mRNA. Interestingly, miRNA encoded by the same polycistronic cluster tend to target the same gene or different genes in the same pathway, enhancing its regulatory role. Additionally, some mRNA presenting

longer 3'UTRs with more miRNA target sites may constitute relevant regulation points in cell networks, and few miRNA are predicted to account for regulation of the majority of network regulation sites. Thus, it is possible that specific gene expression signatures could be established and maintained by a small number of miRNA [39]. It should also be considered that the biological effect of a specific miRNA is context-dependent, due to differential target gene expression in different cell types, and it is caused not solely by the direct effect on target gene expression, but also by indirect changes in gene expression pattern due to miRNA targeting of transcription factors. Indeed, many miRNA target genes are known to be transcription factors, some transcription factors tend to regulate miRNA more tightly than other genes and are more likely to be regulated by these miRNA [39].It was described that gene expression changes after miRNA disruption were mostly caused by transcription alterations due to transcription factor regulation, more frequent and extensive than post-transcriptional changes [40]. Moreover, it was suggested that some miRNA could directly modulate gene expression, possibly by facilitating RNA polymerase binding and activity in specific genes, and by recruiting chromatin modifiers, crosstalking with other epigenetic players [17, 38].

Cancer cells present distinct miRNA patterns compared to normal cells, which might be due to alterations in miRNA expression and/or to dysregulation of miRNA biogenesis pathway [22, 39]. miRNA expression can be affected by the classic factors that influence gene expression. Indeed, miRNA genes are frequently located at fragile sites or genomic regions subjected to mutations (deletions, amplifications or translocations) in cancer [41, 42], and altered transcription factor activity can also change miRNA expression [37]. Additionally, miRNA may be also regulated by epigenetic mechanisms,

especially DNA methylation and histone modification, in a dynamic tissue-specific, miRNA specific and epigenetic effector specific manner [37, 38]. Only about 50% of miRNA genes associate with CpG islands, whereas several miRNA have been reported to be differentially methylated in cancer [43], and histone methylation as well as HDAC overexpression can alter miRNA expression [38]. Moreover, several components of miRNA biogenesis pathway were reported to be dysregulated in cancer: DROSHA and DICER expression levels can be upregulated or downregulated in different cancer models, DGCR8 and Argonaute proteins were reported as upregulated and XPO5 as downregulated in several cancers, and are globally related to an altered miRNA profile, distinct tumour behavior, clinical features or prognosis [37].

In addition to miRNA deregulation, molecular mechanisms to avoid miRNA regulation are also present in cancer cells, as expression of mRNA isoforms with short 3' UTR and consequently less miRNA binding sites, and single-nucleotide polymorphisms (SNPs) in miRNA genes and in genes required for miRNA biogenesis [39].

Deregulated miRNA in RCTs are involved in key cellular pathways, as VHL-HIF network (for instance, miR-30c-2-3p, miR-30a-3p, miR-210, miR-206), TGFβ and epithelial to mesenchymal transition (EMT) (miR-141, miR-200a, miR-200b, miR-200c), and MET and mTOR pathways (miR-21, miR22, miR-221, miR-222, miR-486, miR-23b-3p) (Tables 1-7). These miRNA are altered by SNPs or promoter methylation, as well as by mutations in *DROSHA*, *DGCR8* and *DICER* [22, 44]. Interestingly, specific miRNA are associated with distinct RCT subtypes [45, 46], tumour stage and grade [47], and prognosis [48-50], and, thus, their clinical potential in RCTs is diverse and promising [51].

1.2.2.3 Malignant tumours – renal cell carcinoma

Because ccRCC is the most frequent RCC subtype, and early studies focused only on ccRCC or included series comprising several RCC subtypes but with ccRCC predominance, the genetic and epigenetic knowledge of this subtype is broader than the remaining RCT subtypes. However, in recent years, an increasing number of publications focusing on less frequent RCT subtypes has been noted. This allows for a deeper understanding on renal carcinogenesis and the comparison of molecular mechanism underlying each RCT morphotype.

1.2.2.3.1 Clear cell renal cell carcinoma

Although it was primordially considered that smaller and circumscribed clear cell tumors were benign [4], it is now well established that all enclose metastatic potential, which increases with the growing size of tumours for kidney confined lesions, and even small clear cell tumours can invade renal sinus or perinephric adipose tissue [52, 53]. However, it was only after the Heidelberg classification in 1997 that the “alveolar” clear cell adenoma was dismissed [12].

Morphologically, ccRCC presents acinar, solid alveolar or cystic pattern, characteristically with a regular network of small and delicate blood vessels. It is composed, in routine hematoxylin/eosin sections, by cells with distinct borders and mostly transparent cytoplasm due to accumulation of glycogen and lipids. Some smaller cells with eosinophilic cytoplasm can be observed, especially in higher grade areas, and an eosinophilic variant is also recognized. Nuclei range from round with homogeneous chromatin and inconspicuous nucleoli in low grade tumours, to larger, polymorphic, with coarse chromatin and prominent nucleoli in higher grade tumours

[3, 6, 7]. Ultrastructural features include pinocytotic vesicles, brush-border equivalents and basal infoldings, as proximal tubule epithelial cells [7]. Moreover, large mitochondria with short attenuated cristae and loose matrix are observed in variable number and distribution in tumour cells of the eosinophilic variant [54].

Besides these typical morphological features, chromosomal abnormalities at 3p12-14 (most frequently 3p deletions) have been identified in ccRCC since the late 70s [55]. Subsequent studies reported its absence in other RCC subtypes, especially pRCC [8, 9, 56], and the Heidelberg classification recognized chromosome 3p deletion as a characteristic genetic feature of ccRCC [11, 12]. Additional cytogenetic alterations were consistently described, as chromosome 5q alterations (5q22 trisomy being the second most frequent), and deletion of chromosomes 6q, 8p, 9p and 14q, the last three associated with disease progression, since its frequency is superior in larger and higher grade tumours [10, 12, 57]. Moreover, 1q gain was reported as more frequent in metastatic ccRCC [58]. CGH studies globally confirmed the alterations detected by conventional cytogenetics, additionally identifying loss of 13q and 17p [59].

Interestingly, some genes now known to be altered in ccRCC are located at chromosome 3p, the best characterized and more frequently mutated being von Hippel-Lindau (*VHL*) gene. *VHL* is a tumor suppressor gene identified in the familial cancer syndrome von Hippel-Lindau disease [60] and its inactivation is currently recognized as an early and pivotal event in sporadic ccRCCs pathogenesis [61]. Both genetic and epigenetic alterations were described for *VHL*: nearly 50-75% of sporadic ccRCC display *VHL* mutation, more than 90% loss of heterozigoty and about 5-20% *VHL* promoter hypermethylation [61, 62].

Comprehensive and integrative ccRCC molecular characterization confirmed *VHL* mutation as the most frequent in this histotype, and identified frequent mutations in *PBRM1*, *SETD2*, *KDM5C* and *BAP1*, as well as in *PTEN*, *MTOR* and *TP53* [63-65]. These approaches have also highlighted frequently deregulated genes and altered cell pathways in ccRCC, and portrayed some contributory genetic and epigenetic mechanisms, integrating and expanding several previous reports.

In addition to VHL-HIF1a-hypoxia pathway (Table 1), the most consistently reported deregulated pathways in ccRCC are the chromatin remodeling machinery, including SWI-SINF complex (Table 2) and PI3K-AKT-MTOR. (Table 3).

For VHL-HIF pathway (Table 1), the most frequent alteration is VHL inactivation mostly by deletion or mutation, leading to HIF accumulation and consequent (over)expression of hypoxia-related genes. Epigenetic modulation of other components of this pathway, mostly by promoter methylation or miRNA altered expression, also contributes to pathway deregulation [22].

Mutations in *PBRM1*, *SETD2* and *BAP1*, all located at chromosome 3p, were first described in 2010-2011 and confirmed epigenetic alterations as major events in ccRCC carcinogenesis [33-35]. Additionally, some distinct mutations have been described in epigenetic modulators, as SWI/SIFT components, histone modifying enzymes and DNA methyltransferases and demethylases (Table 2), leading to altered gene expression, specific histone modifications and altered DNA methylation patterns.

Table 1. More frequently described genetic and epigenetic events affecting the components of the VHL – HIF pathway. Main biological effects of each alteration are also briefly described.

Gene (locus)	**Main genetic and / or epigenetic alteration**	**Biological effect**
VHL (3p25.3)	-deletion (78-90%) [63-65] -mutation (50-83%) [63-65] -promoter methylation (5-25%) [22] -miR-21 and miR-92 upregulation [66, 67]	-component of a protein complex (also including elongin B, elongin C, cullin-2) with ubiquitin ligase E3 activity. -*VHL* inactivation leads to absence of ubiquitination and targeting to degradation of target proteins, as HIFs.
HIF-1α (14q23.2)	-deletion (45%) [63] -mutation (0.7%) [33, 63] -miR-210 [68] and miR-122 [69] upregulation -miR-20b downregulation [70]	-induces expression of several target genes, for instance VEGF, involved in hypoxic response -miR-210 upregulation [target genes involved in angiogenesis (EFNA3, PTP1B, HIF-1α), cell cycle and metabolism] [22] -*HIF-1α* increases miR-210 expression and is also a target of miR-210; *HIF-1α* targeting by miR-210 contributes to an oncogenic imbalance between *HIF-2α and HIF-1α* [22].
HIF-2α (2p21)	-miR-30c-2 and miR-30a downregulation [71]	-miR-30c-2 and miR-30a expression is induced by pVHL; loss of VHL leads to *HIF-2α* increase due to absence of ubiquitination and loss of miRNA targeting, contributing to an oncogenic imbalance between *HIF-2α and HIF-1α* [71] -miR-210 upregulation [target genes involved in angiogenesis (EFNA3, PTP1B, HIF-1α), cell cycle and metabolism] [22]
VEGF (6p21.1)	-miR-206 [22] , miR-106a, miR-20b[70] and miR-126 [72] downregulation -miR-15a, miR-34a, miR-106b upregulation [70]	- increased expression of VEGF is pro-angiogenic
TCEB1 (8q21.11)	-mutation (1-3.3%) [63, 64] -*TCEB1* mutation is associated with loss of chromosome 8 (bialelic inactivation) [64]	-component of the transcription factor B (SIII) complex, which activates elongation by RNA polymerase II. -component of the VHL complex; *TCEB1* inactivation leads to absence of ubiquitination of VHL-bound HIF proteins [64]. -mutually exclusive mutations in *TCEB1* and *VHL* [64].

Not surprisingly, a strong link between hypoxia and histone modifications has been disclosed [73], paralleling the two more frequently altered cellular mechanisms by genetic and epigenetic changes in ccRCC, and some histone modifications and altered histone modifying enzymes levels have been associated with ccRCC prognosis [74].

Table 2. More frequently described genetic and epigenetic events affecting chromatin remodelers, histone modifying enzymes and DNA methyltransferases. Main biological effects of each alteration are also briefly described.

Gene (locus)	**Main genetic / epigenetic alteration**	**Biological effect**
SETD2 (3p21.31)	-mutation (4-12%) [33, 63, 64] -deletion (76%) [65]	-histone methyltransferase, catalyzes trimethylation of lysine-36 of histone H3 (H3K36Me3), associated with active chromatin and reduced CpG methylation [22] -*SETD2* mutation associated with loss of DNA methylation at non-promoter regions [22, 63] -*SETD2* loss could contribute to genomic instability (H3K3Me3 required to ATM and TP53 activation of DNA damage checkpoint) [22]
BAP1 (3p21.1)	-mutation (10%) [63, 64] -deletion (75%)[65] -miR-200b and miR-429 downregulation [70]	-ubiquitin C-terminal hydrolase -binds BRCA1 (DNA damage response), E2Fs transcription factors, E2F target gene promoters (cell cycle) [22] -BAP1-mutated tumors present E2F and PRC2 target genes downregulation and increased EZH2 expression [22, 64] -BAP1 inhibits mTOR activation by AKT; BAP1 inactivation associates with mTOR activation [22]
KDM5C / JARID1C (Xp11.22)	-mutation (3.4-7%) [33, 63, 64] -deletion (54%) [65]	-histone demethylase (H3K4Me3 demethylase), inhibits target genes by removing the active chromatin mark H3K4Me3 [22] -induced by HIF; may be involved in regulating HIF target genes' expression levels [22]
KDM6A / UTX (Xp11.3)	-mutation (1-3%) [33, 63]	-histone demethylase (H3K27Me3 demethylase), removes the repressed chromatin mark H3K27Me3; *KDM6A* loss of function might lead to gene downregulation [22]
SIRT1 (10q21.3)	-miR-34a upregulation [70]	
DNMT3B (20q11.21)	-miR-148a upregulation [70]	-alteration in DNA methylation
TET2 (4q24)	-mutation (6%) [64] -deletion (10%) [64] -miR26a downregulation [75]	-alteration in DNA demethylation -histone O-GlcNAcylation during gene transcription [64]
SWI/SNF complex (functional interactions between distinct SWI/SNF complexes and associated co-factors regulate gene-specific transcription; non mutually exclusive mutations in SWI/SNF complex proteins in 58% of ccRCC [64, 76]**)**		
PBRM1 (3p21.1)	-deletion (73-92%) [63, 65] -mutation (26-33%) [63-65]	-regulates expression of genes involved in cell proliferation (p21), cell adhesion and cell signaling (E-cadherin) -mutations (mostly inactivating) associated with p21 downregulation, increased cell proliferation and migration [22], and upregulation of hypoxia-related genes [64].
ARID1A (1p36.11)	-mutation (2-3%) [63, 64] -mutation (3%)[63]	-competition between ARID1A and other ARIDs [76]
ARID1B (6q25.3)	-deletion (20%) [64]	-associated with gene repression, cooperates with ARID2 [76] -represses Wnt/β-Catenin signaling [77]
ARID3B (15q24.1)	-miR-125a downregulation [70]	
SMARCA4 (19p13)	-mutation (2%)[63]	

Table 3. More frequently described genetic and epigenetic events affecting PI3K/AKT/*mTOR* pathway. Main biological effects of each alteration are also briefly described.

Gene (locus)	**Main genetic / epigenetic alteration**	**Biological effect**
SPINT2 (19q13.2)	-methylation (30%) [78]	-inhibits activation of MET by HGF; MET activates mTOR through PI3K – AKT activation; downregulation due to methylation leads to activation of mTOR [22]
EGFR (7p11.2) ***FGFR3*** (4p16.3) ***IGF1R*** (15q26.3)	-mutation (1%) each [64]	-mTOR activation through PI3K – AKT activation
ERBB2 (17q12)	-miR-125a and miR-125b-2 downregulation [70]	
ERBB3 (12q13.2)	-mutation (1%) each [64] -miR-125a downregulation [70]	
ERBB4 (2q34)	-miR224 upregulation [79]	
RACK1 (5q35.3)	-copy number gain (7%) [63]	
FGFR4 (5q35.2)	-mutation (1%) [64] -copy number gain (65%) [63, 64]	
PTEN (10q23.31)	-mutation (2-4%) [63, 64] -deletion (18%) [63] -miR-21 upregulation [70] -miR-214 and miR-494 downregulation [70]	-mTOR inhibition through PI3K – AKT2 inhibition -loss of PTEN causes mTOR activation
PIK3CA (3q26.32) ***PIK3CB*** (3q22.3) ***PIK3CG*** (7q22.3)	-mutation (3-5%) [63, 64] -mutation (1%) [64] -mutation (2%) [64]	-mTOR activation through AKT2 activation
AKT2 (19q13.2)	-mutation (2%) [64] -copy number gain (2%) [63]	-mTOR activation
RPS6KA2 (6q27) ***RPS6KA3*** (Xp22.12) ***RPS6KA5*** (14q32.11) ***RPS6KA6*** (Xq21.1)	-mutation (3%) [64] -mutation (1%) [64] -miR-148 upregulation [70] -mutation (1%) [64]	-mTOR activation through TSC1/TSC2 inhibition
TSC1 (9q34.13) ***TSC2*** (16p13.3)	-mutation (2%) [64] -deletion (2%) [63]	-mTOR inhibition
SQSTM1 (5q35.3)	-amplification (7%) [63]	-mTOR and nuclear factor kappa-B (NF-kB) signaling activation
MTOR (1p36.22)	-mutation (6%) [63-65] -miR-99a downregulation [80]	-cell proliferation -HIF-regulated transcription, some EMT pathways in RCC, IL4 and IGF1 signaling [65]

For PI3K-AKT-MTOR pathway (Table 3), several genes were found to be altered by genetic mutations, promoter methylation and/or microRNA deregulation, mostly with

low frequency, but cumulative alterations might be significant for ccRCC carcinogenesis [64].

Other frequently reported deregulated pathways in ccRCC include Wnt-beta-catenin (Table 4), TGFβ and EMT (Table 5), and p53 signaling, cell cycle regulation and apoptosis (Table 6). These pathways, similarly to PI3K/AKT/*mTOR* pathway, depict numerous epigenetic alterations, mostly promoter methylation and microRNA deregulation, in addition to some low-frequency genetic mutations.

Table 4. More frequently described genetic and epigenetic events affecting WNT – β-catenin pathway. Main biological effects of each alteration are also briefly described.

Gene (locus)	**Main genetic / epigenetic alteration**	**Biological effect**
CDK8 complex	-mutations in GCN1L1, MED12, CCNC (16%) [65]	-CDK8 complex regulates β-catenin-driven transcription [65]
MACF1 (1p34.3)	-mutation and methylation (<11%) [65]	-activation of Wnt/β-catenin signaling in ccRCC [65]
SFRP1 (8p12-11.2)	-methylation (50%) [78] -miR-34a upregulation [79]	-SFRPs bind to Wnt receptor but lack transmembrane and cytoplasmic domain needed for signal transduction and prevent its binding to FZ, thus downregulating Wnt/β-catenin signaling; downregulation due to methylation leads to activation of Wnt/β-catenin signaling [22, 65]
SFRP2 (4q31.3)	-methylation (56%) [78]	
SFRP4 (7p14-13)	-methylation (56%) [78]	
SFRP5 (10q24.1)	-methylation (59%) [78]	
WIF1 (12q14.3)	-methylation (76%) [78]	-inhibit Wnt/β-catenin signaling by binding to Wnt receptor; downregulation due to methylation leads to activation of Wnt/β-catenin signaling [22]
IGFBP1 (7p14-12)	-methylation (35%) [78]	-inhibit Wnt/β-catenin signaling by binding to LRP5, LRP6 and FZ (only *IGFBP1*) subunit of Wnt receptor; downregulation due to methylation leads to activation of Wnt/β-catenin signaling [22]
DKK1 (10q11.2)	-methylation (44%) [78]	
DKK2 (4q25)	-methylation (58%) [78]	
DKK3 (11p15.2)	-methylation (53%) [78]	
SLIT2 (4p15.31)	-methylation (20%) [81]	-SLIT2 binding to ROBO1 activates GSK-3β which leads to degradation of cytoplasmic β-catenin and stabilizes β-catenin and E-cadherin interaction [22]
ROBO1 (3p12)	-methylation (27%) [81]	
CTNNB1 (3p22.1)	-miR1826 downregulation [82]	-nuclear β-catenin location leads to upregulation of target genes as cyclinD1, VEGF, cMYC, cMET [22, 65]

Table 5. More frequently described genetic and epigenetic events affecting TGFβ-TGFR-SMAD, E-cadherin and EMT pathway. Main biological effects of each alteration are also described.

Gene (locus)	**Main genetic / epigenetic alteration**	**Biological effect**
FBN2 (5q23.3)	-mutation (3%) [81] -methylation (40-53%) [81]	-FBN binds TGFβ, preventing its binding to TGFR and induction of ZEB1 and SNAIl expression (via SMAD2-4) and subsequent CDH1 repression [22]
TGFB2 (1q41) ***TGFBR2*** (3p24.1)	-miR-141 downregulation [70] -miR-21 upregulation [70]	-TGFβ activation of TGFR/SMADs/ZEB1-2 pathway inhibits the expression od miR-200 family and miR-30c [22]
ZEB1 (10p11.22) ***ZEB2*** (2q22.3)	-miR200 family (miR-141, miR-200b, miR-200c) downregulation [22, 70]	-miR-200 family inhibits ZEB1 and ZEB2; downregulation of miR-200 family reduces ZEB1-2 inhibition, thus increasing the transcriptional repression of CDH1 [22]
CDH1 (16q22.1)	-methylation (83%) [78]	-pVHL loss causes HIF accumulation, which induces ZEB1 and SNAIL expression that repress CDH1 expression -EMT is promoted by low levels of E-cadherin (encoded by *CDH1*) [22]
PCDH8 (13q14.3) ***NRXN3*** (14q24.3-q31.1) ***CADM1*** (11q23.3) ***CADM2*** (3p12.1)	-methylation (19-62%) [81] -deletion (45%) [63] -miR-15a upregulation [70] -deletion (51%) [63]	-cell adhesion [22]
COL1A1 (17q21.33)	-methylation (65%) [78] -miR-218 downregulation [70]	-collagen family, extracellular matrix
COL1A2 (7q21.3)	-methylation (29%) [83]	
COL14A1 (8q24)	-methylation (44%) [84]	
COL15A1 (9q22)	-methylation (53%) [84]	
LOXL1 (15q24) ***LOX*** (5q23.1)	-methylation (35%) [84] -miR-149 downregulation [79]	-establishes crosslink between collagen and elastin [22]

These cellular pathways interact with each other, and also with other cellular processes, which are also affected by additional genetic and epigenetic alterations, such as:

- energy homeostasis: *FLCN*, methylated in 21% [78]; *SDHD* and *ISCU1/2*, targeted by miR-210 (upregulated) [22, 68]; *UQCRH*, methylated in 36% [63]; *IDH2*, with copy number gain (5%) [63];

Table 6. More frequently described genetic and epigenetic events affecting p53 signaling, cell cycle checkpoints and apoptosis, altered in approximately 40% of ccRCC. Main biological effects of each alteration are also briefly described.

Gene (locus)	**Main genetic / epigenetic alteration**	**Biological effect**
ATM (11q22.3)	-mutation (2-3%) [63, 64] -copy number gain (28%) [65]	-p53 activation through *CHEK2* [64]
CHEK2 (22q12.1)	-mutation (1%)[64]	-p53 activation [64]
TP53 (17p13.1)	-mutation (3%)[64], (2%)[63] -miR-125a downregulation [70]	-induction of apoptosis, senescence, cell cycle arrest, DNA repair and changes in metabolism [64]
CDKN1A	-miR-21[68] and miR-106a [70] upregulation	-cell cycle regulation
CDKN2A (9p21.3)	-deletion (16%)[64], (32%)[63] -methylation (11%)[81]	-cell cycle regulation
CCNB2 (15q22.2)	-mutation (1%)[63]	-cell proliferation
CCND1 (11q13.3)	-miR-155 [68], miR-16-2, miR-34a, miR-503[70] upregulation	-cell proliferation
CCNF (16p13.3)	-miR-210 upregulation [22]	-cell cycle regulation
MDM2 (12q15)	-mutation (1%)[64] -miR-192, miR-194, miR-215 downregulation [85]	-p53 inhibition [64]
MDM4 (1q32.1)	-copy number gain (14%) [63]	-p53 inhibition [64]
MYC (8q24.21)	-copy number gain (23%)[64], (15%) [63] -miR-135a downregulation [86]	
E2F1 (20q11.22)	-miR-21 and miR-106b upregulation [70]	-cell cycle regulation
E2F2 (1p36.12)	-mutation (1%)[64] -miR-210 upregulation [22]	
E2F3 (6p22.3)	-miR-34a upregulation [70]	
E2F6 (2p25.1)	-miR-193a upregulation [70]	
p14ARF (9p21)	-methylation (36%) [78]	
p16INK4 (9p21)	-methylation (10%) [78]	
RASSF1 (3p21.31)	-methylation (59%) [78]	-regulates cyclin D1 degradation during metaphase and G1/S phase (inhibits proliferation) and activates apoptosis in response to DNA damage via ATM or JNK pathways [22]
BCL2 (18q21.33)	-miR-15a, miR-15b, miR-16-2, miR-34a upregulation [70]	-anti-apoptotic activity
APAF1 (12q23)	-methylation (98%) [78]	-apoptosis initiation (p53 signaling pathway)
DAL1/4.1B (18p11.3)	-methylation (45%) [78]	
DAPK (9q34.1)	-methylation (38%) [78]	-apoptosis induction
MCL1(1q21.2)	-miR-204 downregulation [72] -miR-15a, miR-193a upregulation [70]	-anti-apoptotic activity; induced by PI3K signaling

- ubiquitination: besides *VHL*, *BAP1* and *CUL7* alterations [34], *KEAP1* was found to be mutated in 4.7% of cases [64];
- mRNA processing: *QKI*, deleted in 28% [63], and other genes involved in the later steps of splicing, as the release of introns (for example *DHX38, DHX9, FUS, GTF2F2, HNRNPM, PCBP1, PRPF4, SNRPG, YBX1*), 3' end processing machinery (*CPSF2, PAPOLA, PCF11*) and mRNA export to the cytoplasm (*AAAS, NUPs, RANBP2, RBM8A, SEH1L*) [64];
- JAK/STAT pathway: *JAK2*, with copy number gain (3%) and targeted by miR-135a-2 downregulation [70];
- carcinogen-induced damage and drug resistance mechanisms: *FHIT*, deleted in 80% [63] and methylated in 53% [78]; *ABCA13*, mutated in 45% [65]; *MDR1*, methylated in 87% [78].

These altered pathways are associated to changes in ccRCC gene expression. Indeed, distinct gene expression patterns were disclosed between *VHL*-mutated and wild-type (105 genes, involved in disulfide bonds, signaling, extracellular region and *EGF*-like domains), *PBRM1*-mutated and wild-type (484 genes, involved in glycoproteins, signaling, secretion and extracellular region), *SETD2*-mutated and wild-type (166 genes, involved in prothrombin activation pathways, RAB domain proteins, zinc finger regions), and *BAP1*-mutated and wild-type (1136 genes, involved in cell adhesion, glycoproteins, signaling and membrane proteins)[63]. Moreover, the vast majority of *PBRM1, SETD2* and *BAP1* mutations were observed in *VHL*-mutated ccRCC, with *PBRM1* mutations being mutually exclusive with *BAP1* mutations [64]. Recent reports

suggest a multistep carcinogenic process in ccRCC, or at least in a subset of ccRCC, that starts with *VHL* inactivation and HIF signaling activation, followed by *PBRM1* inactivation and further amplification of HIF and STAT3 signaling, associated with increased mTOR signaling [87, 88]. Conversely, *BAP* mutations were associated with worst prognosis [64].

These mutational patterns might be associated with distinct DNA methylation, histone modifications and miRNA deregulation patterns, and loss of methylation in non-promoter gene regions has been already associated with SETD2 mutation [63, 89]. These changes might also associate with specific alterations in gene expression. Indeed, specific mRNA and miRNA clusters were identified in ccRCC [90], associated with mutation profile or methylation pattern [63, 91], with some consistency between reports [63, 92]. Additional evidence points to a correlation between gene expression and chromatin accessibility changes, independent of promoter methylation status. Interestingly, these epigenetically activated genes functionally mimic the activation of HIF signaling pathway, regardless of VHL mutational status [93].

Understanding the specific pattern of these deregulated pathways in each ccRCC, in addition to tumor heterogeneity [94] and mutational and epimutational evolutionary history of the tumor [95], is highly pertinent as it seems to portend clinical relevance in regard to prognosis [96], metastatic potential [47, 97] and targeted therapy selection and/or response [98].

1.2.2.3.2 Papillary renal cell carcinoma

Papillary growth pattern was early described in RCTs, and the first morphological classifications considered tumours with papillary architecture as a distinct category of

either benign or malignant renal cell neoplasms, but these categories were thought to be solely descriptive [4]. The first evidence of differences in prognosis among RCC histotypes was recognized for pRCC in 1976, when a improved survival was noted for tumors with papillary architecture, even when only stage I tumors were compared [99], strengthening the recognition of pRCC as an independent entity.

Papillary RCCs usually demonstrate pushing borders and a pseudocapsule, friable consistency, and are whitish-gray to yellowish or brown, sometimes with hemorrhagic areas, fibrosis and necrosis [3, 7]. Microscopically, tubulopapillary architecture, macrophages in papillae stalks and presence of abundant necrosis in some tumors with good prognosis were early reported [99]. Paralleling the morphological recognition of pRCC, called chromophilic by some authors [6], since the early 90s that chromosomal alterations have been consistently reported in pRCC, more frequently trisomy of chromosomes 7 and 17 and loss of chromosome Y, also in addition to trisomy of chromosomes 3q, 8, 12, 16 and 20 [8, 9, 55, 100], and this accumulating evidence was recognized by the Heidelberg classification [11, 12].

Subsequently, two morphological variants of pRCC were described, type 1 and type 2 pRCC [101], and recognized as pRCC subtypes in the 2004 WHO classification [13]. Type 1 pRCCs present a papillary or tubular architecture and are composed of small cells with pale to slightly basophilic scant cytoplasm containing free ribosomes, cisternae of rough endoplasmic reticulum and moderate number of small mitochondria; oval nuclei and inconspicuous nucleoli. Cells can be cubical or cylindrical and are arranged in a single layer without stratification. Edema, foamy macrophages and psammoma bodies might be identified in papillary cores [6, 101]. Type 2 pRCCs display larger cells with eosinophilic cytoplasm due to a larger number of mitochondria; large round nuclei

usually centrally located; prominent nucleoli; and nuclear pseudostratification [3, 6, 101]. Nonetheless, some tumors present mixed features and the classification of specific cases might be challenging [102].

Regarding molecular characterization, comprehensive studies [103, 104] confirmed chromosome 7 and 17 trisomy as the most frequent cytogenetic alterations in pRCC, with additional cytogenetic events in a lower percent of cases, including 1p and 9p loss and 12q and 16q gain [105-112]. Moreover, one of the genes located in chromosome 7 and mutated in hereditary papillary renal-cell carcinoma, the proto-oncogene *MET*, was also found to harbor somatic mutations in 13 to 17% sporadic pRCC [104, 113, 114]. *MET* encodes for a receptor tyrosine kinase, and binding to its ligand - HGF - prompts MET receptor dimerization and activation of PI3K-AKT-mTOR pathway, stimulating cell growth, survival and EMT; activation of RAS-RAF-MEK-ERK pathway and subsequent cell proliferation; and also can activate CDC42, increasing cell motility [22]. Besides activating *MET* alterations, additional genetic and epigenetic alterations were reported in other components of this pathway (Table 7). Mutations in MET were found to be mutually exclusive with PTEN, TSC1 and MTOR mutations [104, 113].

Similar to ccRCC, epigenetic deregulation has been implicated in pRCC tumorigenesis, as mutations involving chromatin remodelers and histone modifying enzymes were observed in pRCC, as well [102, 104, 113]:

- Chromatin remodeling complex SWI/SNF: *PRMT1*, mutated in 4% [104]; *ARID1A*, mutated in 5.2% [102]; *ARID2*, mutated in 2.3% [102]. Pathway mutations ranging from 20% (type 1 pRCC) to 27% (type 2 pRCC) [104].
- Histone modifying enzymes: *BAP1*, mutated in 4.7% [102] and *SETD2*, mutated in 6.5-7.5% [102, 113]; *KDM6A* mutation (4%) and in other KDMs (*KDM1A*, *KDM1B*,

KDM4B, *KDM5B*, *KDM5C*, *KDM6B*; mutation <1%) were also reported [104]. Pathway mutations ranging from 35% (type 1 pRCC) to 38% (type 2 pRCC) [104].

Mutations in *PRMT1* and *BAP1* were mutually exclusive, but *PRMT1* mutations were found in *SETD2*-mutated tumors [104].

Table 7. More frequently described genetic and epigenetic events affecting MET-PI3K-mTOR pathway. Main biological effects of each alteration are also briefly described.

Gene (locus)	**Main genetic / epigenetic alteration**	**Biological effect**
SPINT2 (19q13.2)	-methylation (45%) [78]	-inhibits activation of MET by HGF, and subsequent mTOR activation; downregulation due to methylation leads to mTOR activation [22]
MET (7q31.2)	altered (81% in type 1 pRCC) by -mutation (7.6-17.4%) [102, 104, 113] -copy number gain (7q) [104] -alternative splicing (5%) [104] -miR199a (downregulated) [115]	-activates PI3K-AKT-mTOR and RAS-RAF-ERK pathways [22]
ERRFI1 (1p36)	-deletion 1p36 (11.2%) [104]	-inhibits EGFR (that activates mTOR) [104]
PTEN (10q23.31)	-mutation (1.5%) [104, 113]	-mTOR inhibition through PI3K – AKT2 inhibition; PTEN loss causes mTOR activation [22]
RAS (12p12.1)	-mutation (1.5%) [104, 113]	-activates RAF-MEK-ERK pathway, stimulating cell proliferation [22]
RASSF1(3p21.31)	-methylation (75%) [78]	-inhibits RAS [22]
MTOR (1p36.22)	-mutation (1.5%) [113] -miR199a (downregulated) [115]	-cell proliferation; HIF-regulated transcription, some EMT pathways in RCC, IL4 and IGF1 signaling [65]

Other pathways reported to be altered in pRCC include:

- NRF2-ARE pathway, involved in response to oxidative stress: *CUL3*, mutated in 4.1%; *NFE2L2*, mutated in 3.5%; *KEAP1*, mutated in 1.2%; *FH*, mutated in 0.6% [102]. Pathway mutation in 9.3% pRCC [104].

- Hippo signaling pathway: *NF2* is mutated in 4.3% of pRCC [113], always in wild-type *MET* cases [104, 113]. Pathway mutations ranging from 3% (type 1 pRCC) to 10% (type 2 pRCC) [104].
- Cell cycle regulation: *CDKN2A*, inactivated by mutation (0.6-1.7%) [102, 104], promoter methylation (6.2%) and 9p21 loss (8.1%), and targeted by miR-10b (upregulated) [104].

Several studies compared genetic and epigenetic features between type 1 and type 2 pRCC, some focusing on differences in molecular features [105-110] and others suggesting a link or a progression from type 1 to type 2 pRCC [111, 112]. Regarding cytogenetic alterations, namely copy number alterations, some variability was noted among studies, possibly related to morphological case selection and differences in methodology [102]. Notwithstanding, trisomy of chromosomes 7 and 17 was consistently reported in type 1 pRCC, whereas in type 2 pRCC a more heterogeneous list of alterations and worst prognosis was depicted. This pattern was confirmed by recent comprehensive analysis of pRCC [104], reinforcing the idea that type 1 pRCC is a more robust genetic entity and type 2 pRCC encompasses heterogeneous tumors and might be further subdivided. Interestingly, separate studies identified a subgroup of type 2 pRCC (mostly low grade) with genetic and mRNA expression profiles more similar to type 1 pRCC [103, 104, 107]. This subgroup also presented a distinct methylation profile compared to other type 2 pRCCs, some of them with *SETD2* mutation [104]. Additionally, upregulation of miR-210 and let-7c, modulating JAK-STAT signaling (*MYC, STAT2, STAT6*) and p53, was reported in type 1 pRCC [110].

Concerning pathway analysis, type 1 pRCC frequently (81%) presents *MET* (activating) alterations followed by mutations in chromatin modifiers (35%) and SWI/SNF complex

(20%); whereas in type 2 pRCC the most recurrently reported altered pathways are chromatin modifiers (38%), SWI/SNF complex (27%), p53 signaling (*CDKN2A* inactivation in 25%), NRF2-ARE pathway (25%), and Hippo signaling pathway (10%) [104].

Challenging the traditional view of individual gene alterations as driver events in carcinogenesis, one study proposed chromosome-scale copy number changes as the truncal driver event in pRCC, involving simultaneous altered expression of several genes and miRNAs and subsequent sub-clonal acquisition of distinct driver mutations, namely in epigenetic players, such as *BAP1*, *SETD2* and *ARID2* [102] .

Hence, molecular data on pRCC carcinogenesis highlight the role of epigenetic mechanisms and the need to use strict morphological criteria and adequate ancillary techniques for the diagnosis of pRCC, keeping in mind the heterogeneous nature of type 2 pRCC, which might display some degree of morphological overlap with unclassified RCC displaying papillary architecture, MiT family translocation RCC and hereditary leiomyomatosis and RCC associated RCC [3], for instance. In addition, the distinct mutation, gene expression and epigenetic profiles in pRCC might be clinically useful as diagnostic and prognostic biomarkers, and could also be relevant for guided therapy selection [116].

1.2.2.3.3 Chromophobe renal cell carcinoma

Chromophobe renal cell carcinoma was first described in humans in 1985 by Thoenes, as the sporadic counterpart of chemically (nitrosomorpholine)-induced renal neoplasia in experimental models (rats) [4, 117]. These tumors are usually large (average: 7cm), with pushing borders, non-capsulated, beige to brownish, and may present focal

grayish areas [3, 117]. Microscopically, most tumors display large solid sheets of cells not completely separated by vascular septa, but small nests, tubules, microcysts, trabeculae or focal papillae are less frequent architectural features. Large pale cells and small eosinophilic cells, frequently binucleated, with a perinuclear halo and irregular hyperchromatic nuclei with clumped chromatin can be observed [3, 117].

Two morphological variants were described, classic and eosinophilic chRCC. Classic chRCC presents predominantly large cells with light, finely reticular cytoplasm, with a peripheral rim of dense filamentous cytoplasm and a prominent cell membrane ("plant cell"-like). These larger cells usually locate at the periphery of large nests or sheets, and some smaller eosinophilic cells can be identified at the centre [3, 117]. Ultrastructurally, abundant round-elongated or invaginated microvesicles and some larger vesicles are seen mostly around the nucleus, and ribosomes and few mitochondria with variable size and shape, presenting tubulovesicular and, rarely, lamellar cristae, at the periphery [6, 54, 117]. Eosinophilic chRCC is predominantly composed of smaller polygonal cells with markedly eosinophilic and granular cytoplasm, and round nuclei [3, 117]. Ultrastructurally, a higher number of mitochondria with round to oval shape, less size variability, and tubulovesicular or lamellar cristae are present, as well as microvesicles admixed with mitochondria and other organelles [6, 54, 117].

Similar to other RCC, cytogenetic alterations were described in chRCC, the most consistently reported and most frequent being loss of chromosomes 1, 2, 6, 10, 13, 17 and 21, each one present in more than 70% of cases and combined losses in almost all cases [10, 118, 119]. Additional loss of chromosome 3, 5 and 9 was described in 23-40% of cases [119]. Comprehensive studies corroborated these results, describing loss

of most or entire chromosomes 1, 2, 6, 10, 13 and 17 in 86% of classic chRCC, and loss of chromosomes 3, 5, 8, 9, 11, 18 and 21 in 12-58% of classic chRCC. Concerning eosinophilic chRCC, the classic pattern of chromosomal monosomy was reported in 53% of cases, and diploidy in 21% [120]. Concurrently, expression levels of genes located in these chromosomes was found to be globally decreased [119], and altered gene expression implicated endocytosis and vesicle mediated transport (*DOCK1, AP1M2, HOOK2, GDI2, MAL2*) in chRCC tumorigenesis [121]. Interestingly, chRCC present an increased number of microvesicles, as previously described, when compared to other RCTs [54, 117].

Additionally, a lower rate of exomic somatic mutations, compared to other neoplasms as ccRCC, was described [113, 120], as well as the presence of mutations in mitochondrial genome [120, 122, 123]. The main mutated genes are involved in metabolism and mitochondrial function, p53 and mTOR pathways [113, 120] (Table 8) Noteworthy, genes involved in p53 [*TP53* (17p13.1), *CDKN1A* (6p21.2), *RB1* (13q14.2)] and mTOR [*PTEN* (10q23.31), *NRAS* (1p13.2), *MTOR* (1p36.22)] pathways are located in chromosomes frequently (>70%) lost in chRCC [120]. Interestingly, the presence of mutations in mitochondrial genes was not associated with gene expression patterns related to loss of oxidative phosphorylation, suggesting that the increased number of mitochondria observed in chRCC might be a compensatory mechanism, as these mutations occur in heteroplasmy and are most likely inactivating [120].

Besides the low rate of somatic mutations, chRCC also present fewer genes with hypermethylation and more genes with hypomethylation compared to ccRCC [104] and normal renal tissue samples [124]. Both hyper and hypomethylated genes were found altered in less than 50% of cells within the samples, and the most frequently

deregulated cell networks by hypermethylation were tissue development and morphology (13 genes); cell signaling, molecular transport and mineral metabolism (12 genes); and cell cycle, cell death and survival (11 genes) [124]. However, very little is known about epigenetic alterations in chRCC, especially compared to ccRCC.

Table 8. Main altered pathways and genes described in chRCC.

Pathway	Altered gene – mechanism (% chRCC cases)
Metabolism and mitochondrial function	
Somatic genes	-***PDHB*** [favors glycolysis over oxidative phosphorilation] – mutation (4.3%) [113] -***PRKAG2*** [subunit of AMPK, cell metabolism key sensor] – mutation (activating) [113] -**NDUFA4** [complex I respiratory chain] – target of miR-145 (downregulated) [120] -***FAAH2*** [lipid hydrolase] – mutation (4.3%) [113] -***PPARGC1A*** [regulator of mitochondrial biogenesis] – upregulation[120]
Mitochondrial genes (mutation in 20%, with >50% heteroplasmy) [120]	**Electron transport chain complex 1** genes (altered in 18%) -***MT-ND5*** – mutation (9.8%) [120] -***MT-ND1*** – mutation (3.3%) [120] -***MT-ND2*** – mutation (3.3%) [120] -***MT-ND4*** – mutation (3.3%) [120]
Telomere maintenance and DNA repair	-***TERT*** [upregulation] – genomic structural rearrangements involving promoter (10%) [120]
TP53 pathway	-***TP53*** – mutation (21-32%) [113, 120]
mTOR pathway (genomic targeting in 23%) [120]	-***PTEN*** – mutation (6.4-9%) [113, 120] -***TSC1*** – mutation (6%) [120] -***TSC2*** – mutation (6%) [120] - ***mTOR*** – mutation (3%) [120]
Chromatin remodeling	-***ARID1A*** – mutation (4,3%) [113]

Regarding prognosis, chRCC clinical behaviour is mostly favorable, although an aggressive subgroup with metastatic potential and worst prognosis exists [125]. On macroscopic and histological grounds, some overlap might exist between chRCC, mainly the eosinophilic variant, and renal oncocytoma. Together with the globally favorable prognosis of chRCC, these features raised the question of a possible link between chRCC and oncocytoma [4], and highlighted the need to find biomarkers that

might be useful to distinguish them [126, 127]. Indeed, considerable molecular data on chRCC focus on the distinction between chRCC and renal oncocytoma. Several genetic and epigenetic markers were found to be differentially expressed between these subtypes, and distinct panels were proposed to specifically distinguish chRCC from oncocytoma, including gene expression profiles (*API1M2 / MAL2 / PROM2 / PRSS8 / FLJ20171* [121]; *ASB1 / GLYAT / PDZK1IP1 / PLCG2 / SDCBP2* [113]; and HRPT2 / AQP6 / SYNGR3 [128]) and DNA methylation profiles (hypermethylation of *ALCAM* and *TRPC4P* in oncocytoma, and *TFAP2B*, *HOXA9*, *DBC1* and *CACNG7* in chRCC [124]). Additionally, miRNA expression was associated with prognosis (high miR-210 expression and short disease free survival) [129]. Nonetheless, these biomarkers did not reach routine implementation, likely due to lack of validation in large independent cohorts, and to inconsistent immunostaining pattern in clinical samples [126].

1.2.2.4 Benign tumours

1.2.2.4.1 Oncocytoma

Oncocytoma represents 5 to 9% of all renal neoplasms [3, 130], and is a benign renal cell tumor arising from collecting duct intercalated cells, composed of oncocytic cells [131]. Oncocytomas are usually solid and well circumscribed tumors, slightly lobulated, with a yellow-tan to mahogany brown colour and a central scar. However, limited infiltrative growth, focal hemorrhage and eccentric scarring may be seen [3, 7, 127]. Microscopically, oncocytomas present solid and nested architecture, typically with small nests disposed in a loose connective tissue stroma. Tumor cells are large, round, with indistinct borders and a densely granular eosinophilic cytoplasm, round nuclei

typically in the centre of the cell, and small nucleoli. Binucleated cells can also be observed [3, 6, 7, 127]. The cytoplasmic granular and eosinophilic appearance is due to the presence of round and uniform mitochondria, slightly larger than chRCC's mitochondria and with prominent lamellar cristae [6, 54].

Distinct cytogenetic alterations have been described in oncocytomas, the most frequently reported being loss of chromosomes 1 (partially or entirely) and Y, 11q13 rearrangements, and chromosome 14 deletion [9, 10, 132-134]. Somatic pairing of chromosome 19 was also described and associated with deregulated gene expression within paired regions [135]. One of these genes is *ELGN2*, a prolyl-hydroxylase that regulates oxygen dependent HIF degradation. *ELGN2* overexpression in oncocytomas increases HIF degradation and consequent downregulation of hypoxia-inducible target genes [135]. However, it should be kept in mind that mosaicism and a normal karyotype are also common in oncocytomas [8, 133].

Based on the pattern and frequency of these cytogenetic alterations, three oncocytoma groups were purposed: one with chromosome 1 loss, followed by chromosome Y or 14 deletion, another with translocation involving 11q13, and the remaining without recurrent or detectable cytogenetic alterations [133]. The existence of these distinct oncocytoma subtypes was also reported in a comprehensive analysis of oncocytoma, which identified two main subtypes of oncocytomas, one dyploid (type 1) with translocation of 11q13 at the CCND1 locus and cyclin D1 overexpression; and another with loss of chromosomes 1, X or Y, and/or 14 and 21 (type 2) [136].

Regardless of these distinct subtypes, oncocytoma present fewer somatic mutations than other RCTs [113], and mutations in known cancer genes and genes mutated in renal cell tumors (*TP53*, *PTEN*, *FLCN*, *VHL*, *FH*, *SDHB*, *SDHC*, or *SDHD*) were not

detected [136]. Conversely, the main feature of oncocytoma is the presence of inactivating mutations in mitochondrial genes, encoded by the nuclear and mitochondrial genome, is thought to be an early event in tumorigenesis [136]. The previously described mutation in genes encoding electron transport chain complex 1 components (*MT-ND1, MT-ND4* or *MT-ND5*) [137], was found to be recurrent in oncocytoma, the most frequent (>25%) in *MT-COX1, MT-COX2, MT-COX3, MT-ND4, MT-ND5*, and *MT-CYTB* [136]. It was proposed that defects in mitochondrial respiration due to these alterations might activate p53, thus contributing to a metabolic checkpoint that limits tumor progression, and that are also associated with defective Golgi transport and autophagy, leading to the cytoplasmic accumulation of mitochondria, a characteristic feature of oncocytoma [136].

Epigenetic studies on oncocytoma are scarce, with the exception of comparative epigenetic studies with chRCC, which identified global lower percentage of hypermethylation and higher percentage of hypomethylation compared to chRCC [124], and some genes with distinct methylation profile, still lacking independent validation in larger series, as previously described in the chRCC section.

1.2.2.4.2 Papillary adenoma

Papillary adenoma is the most frequent tumour originating from renal epithelial cells [13] and autopsy studies show increasing frequency with age, from 10% between 21 to 40 years old, to 40% between 70 to 90 years old [3].

Presently, only tumours with papillary architecture are included in the renal adenoma category [3]. However, the first reports on RCT classified all small and circumscribed tumours without metastasis as renal adenomas, regardless of the architecture, even

though some authors described papillary and alveolar types. Although Grawitz, in 1883, recommended that only papillary tumours should be classified as renal adenomas whereas alveolar clear cell tumours should be considered as derived from adrenal rests, most authors continued to regard all small localized tumours as renal adenomas irrespective of architectural pattern. A continuum between renal adenoma and carcinoma was postulated, and adenomas were considered the precursor lesions of renal carcinomas by some authors [4]. Tumour size has been established as the main criteria to distinguish adenomas from carcinomas, especially after Bell report in 1938 which classified all renal tumours smaller than 3cm as adenomas regardless of its architecture, and this was adopted by subsequent classification systems including the first WHO classification (1981) [4].

The first classification that restricted renal adenoma to papillary low-grade tumours smaller than 5mm was the Heidelberg classification in 1997 [11], and subsequently the only alteration was in the 2016 WHO classification: the maximum size allowed for papillary adenoma is now 15mm [3]. Moreover, papillary adenomas are cortical tumours, frequently subcapsular, uncapsulated and depicting papillary, tubulopapillary or tubular architecture. Tumour cells have pale cytoplasm, round to oval nuclei with minimal pleomorphism and inconspicuous nucleoli [3].

Papillary adenomas frequently display trisomy of chromosomes 7 and 17, and loss of chromosome Y, similar to pRCC, and thus, cytogenetic analysis may not distinguish papillary adenoma from pRCC [3].

1.2.3. CLINICAL POTENTIAL OF EPIGENETIC ALTERATIONS IN RENAL CELL TUMORS – RATIONALE

Renal cell tumorigenesis is a complex process encompassing both genetic and epigenetic events that concurrently alter key pathogenic cell pathways [22]. Renal cell tumors are not a homogeneous molecular entity, and the most frequent RCTs – ccRCC, pRCC, chRCC and oncocytoma – present a distinct array of genetic and epigenetic alterations, some of them associated with prognosis or therapy response [63, 104, 120, 136].

The advances in kidney cancer treatment have increasingly required an adequate identification of the histological type in pre-surgical biopsies, and more information regarding patient prognosis [138]. As epigenetic alterations are early and ubiquitous events in renal tumorigenesis [15, 22], epigenetic features of renal cell tumors constitute as promising diagnostic and prognostic biomarkers [139] that might provide relevant information for an early diagnosis and personalized therapeutic approach in patients with renal cell tumors [78, 95].

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1.3. THE EPIGENETICS OF RENAL CELL TUMORS: FROM BIOLOGY TO BIOMARKERS

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The epigenetics of renal cell tumors: from biology to biomarkers

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Renal cell tumors (RCT) collectively constitute the third most common type of genitourinary neoplasms, only surpassed by prostate and bladder cancer. They comprise a heterogeneous group of neoplasms with distinctive clinical, morphological, and genetic features. Epigenetic alterations are a hallmark of cancer cells and their role in renal tumorigenesis is starting to emerge. Aberrant DNA methylation, altered chromatin remodeling/histone onco-modifications and deregulated microRNA expression not only contribute to the emergence and progression of RCTs, but owing to their ubiquity, they also constitute a promising class of biomarkers tailored for disease detection, diagnosis, assessment of prognosis, and prediction of response to therapy. Moreover, due to their dynamic and reversible properties, those alterations represent a target for epigenetic-directed therapies. In this review, the current knowledge about epigenetic mechanisms and their altered status in RCT is summarized and their envisaged use in a clinical setting is also provided.

Keywords: epigenetics, kidney, renal cell tumors, DNA methylation, chromatin remodeling, histone post-translational modifications, microRNAs, epigenetic-based therapeutics

INTRODUCTION

Renal cell tumors (RCT) are the most common neoplasms of the kidney in adults, representing 2–3% of all non-cutaneous malignant neoplasms, ranking 14th in incidence for both genders, with a mortality rate of 1.6/100,000, worldwide (Ferlay et al., 2010). RCTs are a very heterogeneous group of neoplasms, ranging from benign to malignant behavior, displaying variable clinical aggressiveness and considerable histopathological diversity (Lopez-Beltran et al., 2009). The most prevalent benign RCT is oncocytoma, whereas among malignant RCTs the most common subtypes are clear cell renal cell carcinoma (ccRCC, 70–75% of cases), papillary renal cell carcinoma (pRCC, 10–15% of cases), and chromophobe renal cell carcinoma (chRCC, 5–10% of cases). Owing to the fact that RCT are mostly asymptomatic at their earliest stages, when curative treatment is more likely to be effective, a significant proportion of cases (up to a third of all RCCs) are diagnosed at a late stage, when the neoplasm has already spread locally or systemically (Lam et al., 2005). On the other hand, an increasing number of RCT is being identified through routine ultrasonography, posing new diagnostic challenges at a pre-operative stage, such as the discrimination between benign and malignant tumors.

Presently, there are no effective screening or early diagnostic tools for RCT that might be used in at-risk groups or in a wider population setting (Rini et al., 2009). However, the development of such tools might have a relevant clinical impact, mainly if they would be based on non-invasive approaches (Scelo and Brennan, 2007). In this regard, cancer-related genetic and/or epigenetic alterations might be used as biomarkers enabling the detection of cancerous cells in clinical samples, thus increasing the ability to identify tumors at their earliest stages (Esteller, 2008). Eventually, those alterations might also prove useful for assessing prognosis, response to therapy, and also for patient monitoring (Esteller, 2008). RCTs display characteristic and often discriminative chromosomal alterations (Baldewijns et al., 2008). These, however, are not easily amenable for detection in clinical samples because it requires tissue culture for karyotyping or FISH analysis using tumor biopsies. On the contrary, epigenetic alterations carry an enormous potential as specific cancer biomarkers (Mulero-Navarro and Esteller, 2008). Furthermore, owing to the reversible nature of epigenetic alterations, these might constitute attractive therapeutic targets, as demonstrated for some hemato-lymphoid neoplasms (Rodriguez-Paredes and Esteller, 2011). Thus, in this review, we address not only the role of epigenetic alterations in renal carcinogenesis, but also their clinical potential in RCT management.

EPIGENETIC MECHANISMS AND THEIR DEREGULATION IN CARCINOGENESIS

Epigenetics might be defined as modifications of the DNA or associated proteins, other than DNA sequence variation itself, that carry information content during cell division (Feinberg and Tycko, 2004). Presently, three main epigenetic mechanisms are recognized: DNA methylation, chromatin remodeling/post-translational histone modifications, and microRNA (miRNA) regulation. DNA methylation and chromatin remodeling/post-translational histone modifications cause changes in chromatin

structure that modulate the accessibility of the nuclear transcriptional machinery to specific DNA sequences. On the other hand, miRNAs interact with mRNAs regulating translation to protein.

DNA METHYLATION

The most extensively studied epigenetic mechanism is DNA methylation which, in mammals, consists on the addition of a methyl group to cytosines preceding guanines (so-called "CpG dinucleotides"), a chemical reaction which is catalyzed by DNA methyltransferases (DNMTs; Goldberg et al., 2007; Lopez-Serra and Esteller, 2008). These CpG dinucleotides are usually clustered in DNA stretches called "CpG Islands," preferentially found at the regulatory regions of genes, i.e., promoter, untranslated regions, and exon 1. In a normal cell, CpG methylation is present mainly in repetitive sequences, retrotransposons, and parasitic sequences, thus contributing to genetic stability, whereas most of the CpG islands within promoters remain unmethylated during development and even after differentiation, with the notable exception of some imprinted genes, genes on the silenced copy of X chromosome in females, and tissue-specific genes (Vaissiere et al., 2008). Gene silencing associated with methylation of promoter regions containing CpG islands may be due to an obstruction of the access of transcription factors or through the promotion of binding of methylcytosine-binding proteins (MBD), which cooperate with DNMTs and histone deacetylases (HDACs; Fraga et al., 2005; Sharma et al., 2010). Recently, an important role in gene expression regulation has been also credited to lower density CpG regions located in the vicinity of CpG islands, the so-called "CpG island shores" (Doi et al., 2009). These are sequences up to 2 kb distant from CpG islands, which are associated with gene expression (Doi et al., 2009; Irizarry et al., 2009). Remarkably, the methylation pattern at CpG island shores is mostly tissue-specific and cancer-associated alterations in those patterns occur at sites that vary normally in tissue differentiation (Irizarry et al., 2009). These observations are in line with the so-called "epigenetic progenitor model of cancer" (Feinberg et al., 2006).

Aberrant DNA methylation is probably the best characterized cancer-related epigenetic alteration and it is considered by some as one of the earliest events in the process of tumorigenesis (Feinberg et al., 2006). Those aberrations include both global and gene-specific hypomethylation as well as gene-specific CpG island promoter hypermethylation (Mulero-Navarro and Esteller, 2008; Sharma et al., 2010). The impact of gene-specific alterations in DNA methylation depends on the function of the affected gene and the type of alteration. Whereas promoter hypomethylation may cause activation of proto-oncogenes, hypermethylation induces silencing of cancer-related genes with tumor suppressive properties (Feinberg et al., 2006; Sharma et al., 2010). On the other hand, decrease of global genome methylation (genome-wide hypomethylation) may lead to genomic instability in repetitive sequences, especially at pericentromeric regions, predisposing to abnormal recombination, facilitating translocations, deletions, and chromosomal rearrangements (Ehrlich, 2005; Mulero-Navarro and Esteller, 2008; Sharma et al., 2010). It was also demonstrated that aberrant methylation may affect large extensions of DNA in cancer cells, resulting in extensive epigenetic reprogramming of entire chromosomal regions (Frigola et al., 2006).

CHROMATIN REMODELING AND HISTONE MODIFICATIONS

Chromatin remodeling refers to covalent and non-covalent modifications of histones, the proteins that form the core of nucleosomes which are the basic unit of chromatin. The N-terminal tails of histones protrude from the nucleosome and may be subjected to a wide range of post-translational covalent changes (methylation, acetylation, ubiquitylation, sumoylation, and phosphorylation) of specific amino acid residues and these are involved in regulation of transcription (Kouzarides, 2007a). This set of histone modifications – the so-called "histone code" – determines the configuration of chromatin, adjusting the accessibility to effector proteins (Kouzarides, 2007a). For instance, whereas lysine acetylation is associated with active transcription, lysine methylation may lead to transcription activation or repression depending on the residue affected and the degree of modification (Kouzarides, 2007a). For example, trimethylation of lysine 4 on histone H3 (H3K4me3) is associated with active transcription, whilst H3K27me3 and H3K9me3 are the two chief repressive marks (Kouzarides, 2007a). There is a large number of enzymes involved in the addition or removal of the covalent modifications, including histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases (HMTs), and demethylases (HDMs; Kouzarides, 2007a,b; Shi, 2007). Besides their important role in regulation of gene expression in somatic cells, covalent histone modifications are also critical for embryonic stem cell (ESC) development and differentiation (Kouzarides, 2007a,b; Shi, 2007). Indeed, ESCs display bivalent chromatin domains which provide epigenomic plasticity during normal development. Those bivalent chromatin marks consist on the simultaneous occupancy of trimethylated histone H3 lysine 27 (H3K27me3, a repressive mark) and of di- and trimethylated histone 3 lysine 4 (H3K4me2 and H3K4me3, which are both active marks) at the promoters of genes encoding transcription factors that regulate developmentally important genes (Mikkelsen et al., 2007). Thus, neoplastic transformation would be associated with an alteration of these marks, leading to silencing of tumor suppressor genes (TSG; through the loss of H3K4me2 and H3K4me3) with associated promoter hypermethylation and/or activation of proto-oncogenes with concomitant loss of methylation (through loss of H3K27me3; Bloushtain-Qimron et al., 2008; Kondo et al., 2008). On the other hand, non-covalent mechanism involved in chromatin remodeling include alterations in nucleosome positioning and incorporation of histone variants (Henikoff, 2008). It should also be emphasized that DNA methylation and histone modifications work in concert to achieve stable patterns of gene expression (Ballestar and Esteller, 2005; Vaissiere et al., 2008). Indeed, nucleosome remodeling may be involved in inappropriate gene silencing through the cooperation with aberrant DNA methylation and repressive histone covalent modifications (Lin et al., 2007). Concerning histone modifications, global loss of both acetylation of lysine 16 (H4K16ac) and of trimethylation of lysine 20 of histone H4 (H4K20me3) are commonly found in cancer cells, usually in association with DNA hypomethylation at repetitive DNA sequences (Fraga et al., 2005). Loss of H4K16ac correlates with gene silencing (Kapoor-Vazirani

et al., 2008), but H4K20me3 is normally found in constitutive heterochromatin regions and other regions that contain silenced genes (Fullgrabe et al., 2011). Thus, it seems paradoxical that, in cancer development, the presence of H4K20me3 is also associated with local silencing of genes (Fullgrabe et al., 2011). In addition, altered methylation patterns of H3K9 and H3K27 have also been found in cancer cells (Nguyen et al., 2002; Valk-Lingbeek et al., 2004). Those modifications, which result in inappropriate gene silencing, are accomplished by histone-modifying enzymes. The expression of some of those enzymes, most notably of HDAC1 and EZH2, is upregulated in several cancers and this is thought to contribute decisively for cancer initiation and progression (Halkidou et al., 2004; Valk-Lingbeek et al., 2004; Song et al., 2005). In this regard, HDMs, like LSD1 which targets methylated H3K4 and H3K9, seem also to play an important role in some cancer models (Metzger et al., 2005; Schulte et al., 2009).

MICRORNAs

MicroRNAs constitute a class of small (∼18–25 nt in length), non-coding RNAs, which are synthesized (pri-miR) and processed in the nucleus (pre-miR), and subsequently in the cytoplasm (mature miR), to finally incorporate the RISC (RNA-induced silencing complex) protein complex (Garzon et al., 2009; Guil and Esteller, 2009). MicroRNAs interact with the 3′UTR of target mRNAs, inducing translational repression or degradation of the latter (Guil and Esteller, 2009). Interestingly, a specific miRNA may regulate multiple mRNAs in the same manner that a single mRNA may be targeted by multiple miRNAs (Shenouda and Alahari, 2009). Moreover, a large proportion of human gene transcripts (estimated between 30 and 70%) are known to be regulated by miRNAs, and these display temporal and tissue-specific patterns. Thus, miRNAs are one of the main classes of regulatory human genes, involved in multiple important cell processes, including cell proliferation, apoptosis, differentiation, development, and metabolism (Guil and Esteller, 2009).

The altered expression of miRNAs in cancer has been more recently documented (Lu et al., 2005). Owing to their role as regulators of mRNA expression, they may act as oncogenes (overexpressed miRNAs that target TSG) or TSG (downregulated miRNAs targeting proto-oncogenes; Guil and Esteller, 2009). Examples of the former include miR-21, targeting *PTEN* (Chan et al., 2005), whereas miR-15 and miR-16, which target *BCL2*, and let-7, targeting *RAS*, typify the latter (Zhang et al., 2007; Ventura and Jacks, 2009). The mechanisms that lead to altered miRNA expression are similar to those of regular genes and include amplification, deletion, mutation, and promoter hypermethylation (Guil and Esteller, 2009; Dudziec et al., 2011).

EPIGENETIC ABERRATIONS IN RENAL CELL TUMORS AND CLINICAL APPLICATIONS

As mentioned in the previous section, the delicate epigenetic homeostasis that characterizes normal cells is frequently disturbed in cancer. Indeed, when compared to genetic alterations such as point mutations, deletions, and amplifications, epigenetic events seem to occur much more frequently and at earlier stages (Sharma et al., 2010; Berdasco and Esteller, 2011). These characteristics not only endow epigenetic alterations a critical role in tumorigenesis, but they also set the basis for their use as cancer biomarkers for early detection, diagnosis, assessment of prognosis, patient monitoring, and prediction of response to therapy, as previously stated (Sharma et al., 2010; Berdasco and Esteller, 2011). In the next sections, the role of each epigenetic mechanism in renal cell tumorigenesis, as well as its potential use as biomarker and therapeutic target, will be discussed.

DNA METHYLATION

Most studies that have investigated promoter hypermethylation in RCT used either a candidate-gene approach (i.e., cancer-related genes deregulated through this mechanism in other malignancies and/or genes known to be mutated in familial RCC) or a functional epigenomic approach, based on genome-wide CpG methylation analysis platforms (e.g., CpG island arrays). **Table 1** provides an overview of the most commonly methylated gene promoters in RCTs according to the cellular pathway in which they are involved. Studies using the candidate-gene strategy disclosed promoters with a high frequency (>70% of cases) of hypermethylation, which include *APAF1* (Christoph et al., 2006a,b), *MDR1* (Costa et al., 2007), and *PTGS2* (Costa et al., 2007), those with intermediate frequency (70–20%), like *RASSF1A* (Battagli et al., 2003; Dulaimi et al., 2004; Gonzalgo et al., 2004; Hoque et al., 2004; Costa et al., 2007; Peters et al., 2007), *CDH1* (Esteller et al., 2001; Morris et al., 2003; Hoque et al., 2004; Costa et al., 2007), *DAPK1* (Morris et al., 2003; Christoph et al., 2006a), *KRT19* (Paiva et al., 2011), *TIMP3* (Esteller et al., 2001; Battagli et al., 2003; Dulaimi et al., 2004; Hoque et al., 2004; Costa et al., 2007), *UCHL1* (Kagara et al., 2008; Seliger et al., 2009), *PCDH17* (Costa et al., 2011), and *TCF21* (Costa et al., 2011), and infrequently methylated (<20%) such as *APC* (Esteller et al., 2001; Dulaimi et al., 2004; Gonzalgo et al., 2004; Hoque et al., 2004; Costa et al., 2007), *CASP-8* (Morris et al., 2003; Christoph et al., 2006b), *CDH13* (Morris et al., 2003), *GSTP1* (Esteller et al., 2001; Dulaimi et al., 2004; Hoque et al., 2004; Costa et al., 2007), *JUP* (Breault et al., 2005), *MGMT* (Esteller et al., 2001; Morris et al., 2003; Dulaimi et al., 2004; Hoque et al., 2004; Costa et al., 2007), $p14^{ARF}$ (Esteller et al., 2001; Dulaimi et al., 2004; Hoque et al., 2004; Costa et al., 2007), $p16^{INK4a}$ (Herman et al., 1995; Esteller et al., 2001; Morris et al., 2003; Dulaimi et al., 2004; Hoque et al., 2004; Costa et al., 2007), *RARβ2* (Battagli et al., 2003; Morris et al., 2003; Dulaimi et al., 2004; Hoque et al., 2004; Costa et al., 2007), and *VHL* (Herman et al., 1994; Clifford et al., 1998; Dulaimi et al., 2004; Banks et al., 2006).

Moreover, because the frequency of point mutations affecting genes involved in familial RCC is relatively low in the sporadic cases, except for *VHL*, the relevance of aberrant promoter methylation in RCC carcinogenesis has been highlighted (McRonald et al., 2009). Indeed, several genes, like *RASSF1A*, *SFRP1*, *DAPK1*, and *SPINT2*, were consistently found to be silenced by promoter hypermethylation but rarely mutated in sporadic RCC (Morris et al., 2011).

In this setting, functional epigenomic approaches emerged as efficient strategies to identify genes whose expression was silenced by promoter methylation in RCC, through the identification of genes re-expressed after treatment with demethylating drugs in RCC cell lines and further validated in primary tumor samples.

Table 1 | Genes frequently methylated in renal cell tumors, according to their function/pathway.

Pathway	Gene	Designation	Frequency (%)	Reference
Hormonal response	*ESR1*	Estrogen receptor 1	69–70	Hoque et al. (2004), Costa et al. (2007)
	ESR2	Estrogen receptor 2	51–53	Hoque et al. (2004), Costa et al. (2007)
	RARβ2	Retinoic acid receptor β2	0–53	Battagli et al. (2003), Morris et al. (2003), Dulaimi et al. (2004), Hoque et al. (2004), Costa et al. (2007)
Signal transduction	*DKK2*	Dickkopf 2	58	Hirata et al. (2009)
	DKK3	Dickkopf 3	50	Urakami et al. (2006)
	RASSF1A	Ras association domain family protein 1 isoform A	21–88	Battagli et al. (2003), Dulaimi et al. (2004), Gonzalgo et al. (2004), Hoque et al. (2004), Costa et al. (2007), Peters et al. (2007)
	SFRP1	Secreted frizzled-related protein 1	34–80	Urakami et al. (2006), Costa et al. (2007), Dahl et al. (2007), Gumz et al. (2007), Morris et al. (2010)
	SFRP2	Secreted frizzled-related protein 2	53	Urakami et al. (2006)
	SFRP4	Secreted frizzled-related protein 4	53	Urakami et al. (2006)
	SFRP5	Secreted frizzled-related protein 5	57	Urakami et al. (2006)
	WIF	Wnt inhibitory factor	73	Urakami et al. (2006)
Tumor invasion	*CDH1*	E-cadherin	11–59	Esteller et al. (2001), Morris et al. (2003), Dulaimi et al. (2004), Hoque et al. (2004), Costa et al. (2007)
	JUP	Junction plakoglobin	91	Breault et al. (2005)
	PCDH8	Protocadherin 8	58	Morris et al. (2011)
	PCDH17	Protocadherin 17	61	Costa et al. (2011)
	SLIT2	Slit homolog 2 (*Drosophila*)	25	Astuti et al. (2004)
	TIMP3	TIMP metallopeptidase inhibitor 3	15–78	Esteller et al. (2001), Battagli et al. (2003), Dulaimi et al. (2004), Hoque et al. (2004), Costa et al. (2007)
Angiogenesis	*GREM*	Gremlin1	20–55	Morris et al. (2010), van Vlodrop et al. (2010)
	COL15A1	Collagen type XV alpha-1	53	Morris et al. (2010)
	COL1A1	Collagen type I alpha-1	56	Ibanez de Caceres et al. (2006)
Apoptosis	*APAF1*	Apoptotic protease activating factor 1	97–100	Christoph et al. (2006a,b)
	DAL1/4.1B	Differentially expressed in adenocarcinoma of the lung	47	Yamada et al. (2006)
	DAPK	Death-associated Kinase	33–41	Morris et al. (2003), Christoph et al. (2006a)
Others	*FHIT*	Fragile histidine triad	52	Costa et al. (2007)
	MDR1	Multidrug resistance receptor 1	86	Costa et al. (2007)
	PTGS2	Prostaglandin endoperoxidase synthase 2	94	Costa et al. (2007)
	TCF21	Transcription factor 21	61	Costa et al. (2011)

This strategy allowed for the discovery of more than 10 candidate TSG in RCC: *SPINT2* (Morris et al., 2005), *IGFBP1*, *IGFBP3*, *COL1A1* (Ibanez de Caceres et al., 2006), *UCHL1* (Kagara et al., 2008), *CXCL16*, *KTN19* (Morris et al., 2008), *BNC1*, *COL14A1*, *SFRP1*, *CST6*, and *PDLIM4* (Morris et al., 2010). Furthermore, gene promoters found to be methylated in cell lines but not in primary tumors (e.g., *SST*, *PTGS1*, *ISG15*, and *THY1*) might be important for cancer progression, whereas upregulated genes following treatment of cell lines with demethylating drugs but without promoter methylation (e.g., *BAP*, *IGSF4*, *RRM2*, *PMAIP1*, *Claudin1*, and *ICAM*), might be reactivated due to changes in promoter methylation status at upstream regulators (Morris et al., 2008). The identification of new candidate TSG may also be accomplished using high-throughput CpG methylation analysis platforms (McRonald et al., 2009) or by isolating methylated DNA by immunoprecipitation (methylated DNA immunoprecipitation, MeDIP) which is then used to perform whole-genome microarray analysis (Morris et al., 2011). These strategies enabled the identification of several new candidate TSG in RCC, including *HTR1B*, *CALCA*, *IGFBP2*, *SOX17*, *COL1A2*, *BMP4*, *HS3ST2*, *FRZB*, *TAL1*, *MCM2*, *KCNK4*, *HOXC6*, *PITX2*, *SEPT5*, *IRF7*, *CCNA1*, *HOXA11*, *TERT*, *TMEFF2*, *EPHA3*, *PGF*, *MYOD1*, *MMP2*, *TNFRSF10C*, *PENK*, *EYA4*, *MYLK*, *IRAK3*, *ZNF215*, *SMARCB1*, *TWIST1*, *SCGB3A1*, and *IGFBP7* (McRonald et al., 2009), as well as *ATP5G2*, *PCDH8*, *CORO6*, *KLHL35*, *QPCT*, *SCUBE3*, *ZSCAN18*, *CCDC8*, and *FBN2* (Morris et al., 2011). Deriving from data collected in several studies, a CpG island methylator phenotype (CIMP) for RCC has been suggested (Dulaimi et al., 2004; McRonald et al., 2009), but this topic warrants further investigation in larger series of cases.

Loss of promoter methylation has been seldom reported in RCC, although several gene promoters less frequently methylated in RCC tumor samples compared to normal kidney tissue have been found, including *CARD15* (methylated in 18% of tumors),

HLA-DRA (24%), *SPARC* (34%), *IL8* (35%), *SEPT9* (39%), *HLA-DPB1* (45%), *TNFSF10* (47%), *VAMP8* (50%), *PRKCDBP* (55%), *HLA-DPA1* (56%), *HDAC1* (58%), *BTK* (58%), *S100A2* (60%), *MPO* (61%), *CRK* (61%), *CAPG* (61%), *NEU1* (69%), *ELL* (71%; McRonald et al., 2009), and *CA 9* (Cho et al., 2001).

The accumulating data on gene promoter hypermethylation in RCC supports the development of clinically relevant biomarkers. Interestingly, different RCC subtypes seem to display different gene sets deregulated by promoter hypermethylation (Dulaimi et al., 2004), and a gene panel (*CDH1*, *PTGS2*, and *RASSF2*) intended for the discrimination among the most frequent RCT subtypes in tissue samples has been evaluated (Costa et al., 2007). However, the application of this approach in an early detection setting requires testing of clinical samples obtained by minimally invasive or (ideally) non-invasive means, of which urine and serum stand as the most likely candidates. This has been attempted using a three-gene panel (*APC*, *RARβ2*, *RASSF1A*) which detected RCC with high specificity and sensitivity (Hoque et al., 2004). Moreover, *RASSF1A* promoter methylation might also prove useful for tumor surveillance/monitoring of RCC cancer patients (Peters et al., 2007).

Promoter hypermethylation of some genes has been associated with clinical and pathological features of tumor aggressiveness and also to have prognostic relevance. Thus, aberrant promoter methylation of *APAF1* and *DAPK1* (Christoph et al., 2006a), as well as of *GREM1* (van Vlodrop et al., 2010) was has been associated with aggressive forms of RCC. Moreover, *APAF1*, *DAPK1* (Christoph et al., 2006a), *JUP* (Breault et al., 2005), *PTEN* (Kim et al., 2005), *UCHL1* (Kagara et al., 2008), *DAL1-4.1B/EPB41L3* (Yamada et al., 2006), *BNC1, COL14A1*, and *SFRP1* (Morris et al., 2010) promoter methylation have been associated with poorer survival, and most of them (*JUP, APAF1, DAPK1, PTEN, DAL1-4.1B, BNC1*, and *COL14A1*) retained independent prognostic value in multivariate analysis (Breault et al., 2005; Christoph et al., 2006a; Morris et al., 2010). In addition, a genome-wide methylation profile of ccRCC using bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) and unsupervised hierarchical clustering analysis, found that the clusters obtained for ccRCC tumor tissues predicted recurrence and these were clinicopathological valid since tumors with vascular invasion, renal vein neoplastic thrombi and higher pathological TNM stage clustered together (Arai et al., 2009). Interestingly, DNA methylation status of non-cancerous tissues was similar to that of respective RCC samples, and they were also associated with patient outcome, suggesting an association of RCC prognosis with precancerous molecular alterations (Arai et al., 2009). Finally, it has been also reported that *ABCG2*/*MDR1* gene promoter was methylated in RCC cell lines, and because this gene is associated with resistance of cancer cells to chemotherapeutic agents, this biomarker might become clinically useful for prediction of response to therapy (To et al., 2008).

Due to the association between promoter methylation and gene silencing, inhibition of DMNTs has been proposed as a therapeutic strategy to reactivate dormant genes. The nucleoside analog 5-aza-2′-deoxycytidine (DAC), a DNMTs inhibitor (DNMTi) has been tested in combination with conventional chemotherapeutic agents (e.g., vinblastine or paclitaxel; Takano et al., 2010; Shang et al., 2011) in RCC cell lines. A synergistic effect was observed in both settings, but clinical studies are required to conclusively demonstrate the therapeutic usefulness of DAC in RCC.

CHROMATIN REMODELING AND HISTONE ONCO-MODIFICATIONS

Most studies on chromatin remodeling and histone onco-modifications in RCT have dealt with the relationship with hypoxia, as well as with the prognostic relevance of those alterations and the potential therapeutic use of histone deacetylase inhibitors.

It is widely acknowledged that *VHL* dysfunction is a pivotal event in ccRCC carcinogenesis (Baldewijns et al., 2008), leading to constitutive expression of genes that mediate cellular adaptation to hypoxia. This gene encodes for the VHL protein (pVHL), a substrate recognition component of an E3 ubiquitin ligase complex that targets hypoxia-inducible factors (HIF; e.g., HIF1α and HIF2α) for ubiquitination and proteasome degradation. HIFs are constitutively expressed and under normoxic conditions they are hydroxylated, allowing for their recognition by pVHL and subsequent targeting for degradation. Lack of pVHL leads to increased levels of HIFs and increased expression of genes involved in cellular response to hypoxia, promoting angiogenesis, invasion and metastasis, evasion of cell death, and cellular metabolism (Rathmell and Chen, 2008; Linehan et al., 2010).

Recent evidence unraveled a strong link between hypoxic cellular response and epigenetic regulation, especially histone modifications, albeit in non-RCC cancer cell lines (**Figure 1**; Johnson et al., 2008). Indeed, hypoxia was reported to be associated with widespread repression of total RNA and mRNA synthesis and to induce global histone modifications typically associated with transcriptional repression (loss of H3K9ac, increase in H3K9me2, H3K27me2, H3K9me3, H3K27me3, H3K4me1), but also unpredictably with gene activation (increase in H3K14ac, H4R3me2 which may facilitate acetylation of histones associated with activation of transcription, H3K4me2, H3K4me3, and H3K7me2; Chen et al., 2006; Johnson et al., 2008). Gene-specific histone modifications included a decrease in H3K9ac and an increase in H3K9/27me2 at hypoxia-repressed genes, an increase in H3K9ac and a decrease in H3K9/27me2 at hypoxia-induced genes, and an increase in H3K4me3 and a decrease in H3K27me3 in all hypoxia-responsive genes (activated and repressed), suggesting that repressed chromatin H3K4me3 enriched and H3K27me3 deprived might be rapidly activated when hypoxia is reversed (Chen et al., 2006; Johnson et al., 2008).

One of the mechanisms that might be involved in altered epigenetic landscape due to hypoxic effects is the regulation of Jumonji domain containing histone demethylases by HIF1α, a critical mediator of hypoxic response (Beyer et al., 2008; Pollard et al., 2008; **Figure 1**). Genes encoding Jumonji domain containing 2-oxoglutarate-dependent oxygenases, characterized by a catalytic Jumonji C (JmjC) domain, were defined as a class of HIF-responsive hypoxia-inducible genes (Pollard et al., 2008). Histone demethylases Jumonji domain containing 1A (JMJD1A), Jumonji domain containing 2B (JMJD2B), and Jumonji domain containing 2C (JMJD2C) showed consistent patterns of regulation by hypoxia, especially JMJD1A and JMJD2B, which were found to be induced by HIF1α but not by HIF2α (Pollard et al., 2008). Since

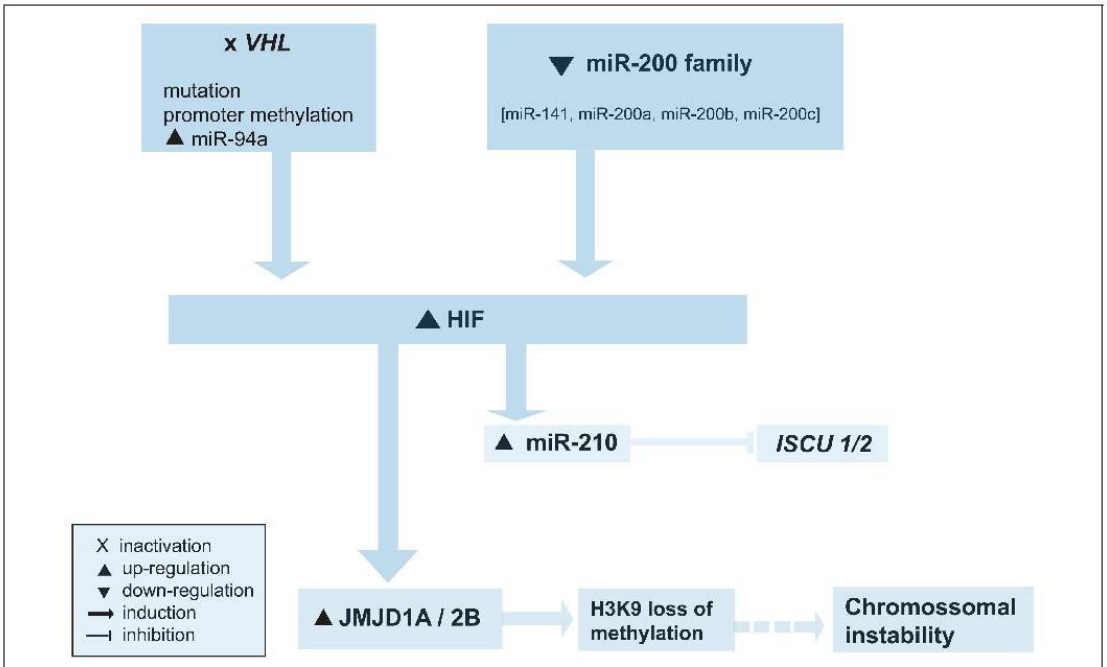

FIGURE 1 | Overview of the epigenetic alterations involved in abnormal hypoxic cellular response. See text for details.

HIF upregulation is a feature of ccRCC, the regulation of histone methylation status, and thus of genetic expression, by JmjC histone demethylases might be another mechanism leading to epigenetic changes in RCC carcinogenesis. Indeed, both JMJD1A and JMJD2B were found to be elevated in a RCC cell line with VHL loss of function (Beyer et al., 2008), and the expression of JMJD1A was reported to be higher in RCC cancer tissue than in adjacent normal renal tissue, mainly in cancer cells surrounding blood vessels, suggesting that JMJD1A is involved in tumor angiogenesis (Guo et al., 2011).

In addition, genes encoding for histone-modifying enzymes have been also reported to be mutated in ccRCC (van Haaften et al., 2009; Dalgliesh et al., 2010; **Table 2**). Specifically, inactivating mutations were described for *SETD2* (H3K36 methyltransferase), *JARID1C*/*KDM5C* (H3K4 demethylase), *UTX*/*KMD6A* (H3K27 demethylase), and *MLL2* (an H3K4 methyltransferase; van Haaften et al., 2009; Dalgliesh et al., 2010). More recently, mutations in the SWI/SNF chromatin remodeling complex gene PBRM1 has been identified in 41% of ccRCCs (Varela et al., 2011). Interestingly, the induction of the H3K9 specific demethylases JMJD1A and JMJD2B, which target H3K9me1/me2 and H3K9me2/me3 respectively, might contribute to an increased mutation rate in tumors with upregulation of HIF, as H3K9 loss of methylation promotes chromosomal instability (Beyer et al., 2008).

The diagnostic potential of histone modifications and/or histone modifiers in RCC has not been explored thus far. They have been, however, proposed as molecular biomarkers of prognosis, easily translated to routine pathology because they may be assessed by immunohistochemistry in formalin-fixed, paraffin-embedded tissue sections. Several histone marks have been associated with poor prognosis in RCC, including low H3K4me2, H3K18ac, and H3K9me2 (Seligson et al., 2009). H3K4me1–3 levels were also found to be inversely correlated with Fuhrman grade, pT stage, lymph node involvement and distant metastases, and an H3K4me score (combining staining levels of H3K4me) was an independent factor for RCC progression-free survival (Ellinger et al., 2010). Similar observations were made for global H3Ac and H4Ac levels, as well as for H3K9Ac levels in RCCs treated with partial nephrectomy (Minardi et al., 2009), whereas H3K18Ac levels were an independent predictor of RCC progression after surgery (Mosashvilli et al., 2010). Concerning histone modifiers, EZH2 was found to be upregulated in ccRCC, but this alteration was unexpectedly associated with a favorable prognosis (Hinz et al., 2009).

Histone onco-modifications might also carry therapeutic implications, as patients with marks of poor prognosis including low levels of H3K4me2, H3K18ac, and H3K9me2 could benefit from innovative treatments with histone deacetylase inhibitors (HDACi) Histone onco-modifications might also carry therapeutic implications, as patients with marks of poor prognosis including low levels of H3K4me2, H3K18ac, and H3K9me2 could benefit from innovative treatments with histone deacetylase inhibitors (HDACi; Seligson et al., 2009). Some preclinical studies on RCC

Table 2 | Histone onco-modifications and deregulated modifiers in renal cell tumors.

Chromatin remodeling alterations		Reference
Histone post-translational modifications	H3K18Ac, H3K4me1, H3K4me2, H3K4me3	Seligson et al. (2009), Ellinger et al. (2010), Mosashvilli et al. (2010)
Histone modifiers	Inactivating mutations of *SETD2*, *JARID1C*, *UTX*, *MLL2* EZH2 upregulation	van Haaften et al. (2009), Dalgliesh et al. (2010) Hinz et al. (2009)

cell lines using the HDACi Vorinostat demonstrated an increase in the anticancer activity of temsirolimus, a mammalian target of rapamycin (mTOR) inhibitor, through survivin downregulation, leading to increased apoptosis, and enhanced inhibition of angiogenesis (Mahalingam et al., 2010). Moreover, the proteasome mediated degradation of Aurora A and Aurora B kinases through inhibition of HDAC3 and HDAC6 by the HDACi LBH589, was found to induce G2-M arrest and apoptosis in RCC, highlighting its potential therapeutic use (Cha et al., 2009). Valproic acid is also an HDACi which causes growth arrest, preventing tumor cell attachment to endothelium and matrix proteins, and blocking integrin-dependent signaling (Jones et al., 2009a). The combination of valproic acid with interferon-alpha enhanced the effects of the former (Jones et al., 2009a,b) and similar synergism was found when combined with AEE788, a multiple receptor tyrosine kinase inhibitor (Juengel et al., 2010). The combination of HDACi and retinoids might also provide an alternative therapeutic strategy because *RARβ2* expression is reduced in RCC, in part owing to gene-specific histone hypoacetylation, and its re-expression is associated with anti-neoplastic effects through the abrogation of retinoid-resistance (Touma et al., 2005; Wang et al., 2005). Interestingly, this synergistic activity with retinoids has been demonstrated for both HDACi MS-275 (Wang et al., 2005) and trichostatin A (TSA; Touma et al., 2005) in RCC cell lines.

MICRORNAs

Deregulation of miRNA expression seems to be pivotal for RCC development and progression (Valera et al., 2011). Indeed, several miRNA have been found to be deregulated in RCTs, although most studies have focused mainly on ccRCC. Upregulation of miR-16 (Jung et al., 2009; Zhou et al., 2010), miR-18a (Neal et al., 2010), miR-20a (Neal et al., 2010), miR-21 (Liu et al., 2010a; Neal et al., 2010; Zhou et al., 2010), miR-34a (Liu et al., 2010a; Zhou et al., 2010), miR-34b (Liu et al., 2010a; Zhou et al., 2010), miR-92a (Valera et al., 2011), miR-155 (Jung et al., 2009; Liu et al., 2010a; Neal et al., 2010; Zhou et al., 2010), miR-185 (Liu et al., 2010a; Zhou et al., 2010), miR-210 (Jung et al., 2009; Liu et al., 2010a; Neal et al., 2010; Zhou et al., 2010), miR-224 (Jung et al., 2009; Liu et al., 2010a) and let-7 i (Neal et al., 2010; Zhou et al., 2010), and downregulation of miR-125b (Liu et al., 2010a; Zhou et al., 2010), miR-141 (Nakada et al., 2008; Jung et al., 2009; Liu et al., 2010a), miR-133b (Liu et al., 2010a; Zhou et al., 2010), miR-200b (Jung et al., 2009; Liu et al., 2010a), miR-200c (Nakada et al., 2008; Jung et al., 2009; Liu et al., 2010a), miR-429 (Jung et al., 2009; Liu et al., 2010a; Zhou et al., 2010), miR-506 (Zhou et al., 2010; Li et al., 2011), miR-508-3p (Zhou et al., 2010; Li et al., 2011), miR-509-5p (Zhou et al., 2010; Li et al., 2011), miR-509-3-5p (Zhou et al., 2010; Li et al., 2011), miR-510 (Liu et al., 2010a; Zhou et al., 2010; Li et al., 2011), and miR-514 (Jung et al., 2009; Liu et al., 2010a; Zhou et al., 2010; Li et al., 2011) are the most consistently reported alterations.

Renal cell tumors display distinct cytogenetic alterations and these might cause miRNA deregulation as matching patterns between deregulated miRNAs and chromosomal aberrations have been reported in ccRCC (Chow et al., 2010a,b). On the other hand, miRNA deregulation might serve as an alternative mechanism for gene expression alterations due to chromosomal aberrations. This is well illustrated by the miR-204/211 family. Whereas in pRCC gain of 3q is a common finding, leading to upregulation of several genes including *C3orf58*, *CCDC50*, *DTX3L*, *PLD1*, *TRIM59*, *ECT2*, *RAP2B*, and *SERP1*, targeted by miR-204/211 (Liu et al., 2010a), in ccRCC miR-204/211 downregulation might be the mechanism causing upregulation of the aforementioned genes, since 3q gain is rare (Liu et al., 2010a). Moreover, it has been postulated that most miRNAs are tandemly clustered (Lee et al., 2002; Seitz et al., 2003), and, accordingly, a co-expression pattern for miRNA families miR-8 (or miR-200), miR-199, and miR-506 has been found in ccRCC (Li et al., 2011). Another interesting example is provided by the miR-506 family members miR-506, miR-508-3p, miR-509-5p, miR-509-3-5p, miR-510, and miR-514, which are downregulated in ccRCC (Zhou et al., 2010). The corresponding genes are tandemly clustered in the fragile site Xq27.3 and their predicted targets are upregulated, including some genes involved in key signaling pathways like *LDHA*, *HK1*, *VEGFB*, and *PSMA1* (Zhou et al., 2010).

Bioinformatics, anti-correlation analysis of miRNA/mRNA levels and functional studies in paired tumorous and normal tissues are also revealing interesting data on cell function alterations due to deregulated miRNA in RCTs. Those have showed that deregulated microRNAs target genes are commonly involved in metabolic (71 target genes of 13 deregulated microRNAs), focal adhesion, cell adhesion molecules and ECM receptor interactions (30 target genes of 25 deregulated microRNAs), cell cycle regulation (24 target genes of 22 deregulated microRNAs), and apoptosis (14 target genes of 11 deregulated microRNAs) pathways in ccRCC (Zhou et al., 2010; **Table 3**).

Furthermore, the miR-200 family is also known to be involved in epithelial-to-mesenchymal transition, and its downregulation might contribute to tumor invasion and metastasis (Liu et al., 2010a; Zhou et al., 2010; Li et al., 2011).

Downregulation of TSG in RCC has been also correlated with upregulation of oncogenic miRNAs. Indeed, elevated miR-185 was correlated with downregulation of *PTEN* (with subsequent activation of PI3K-AKT-mTOR signaling pathway), *PTPN13* (a Fas-associated tyrosine phosphatase that can inhibit PI3K/AKT signaling, induce apoptosis and suppress the cell survival effects

Table 3 | MicroRNA deregulation in renal cell tumors, according to their function/pathway and target genes'.

Pathway	Upregulated	Target gene(s)	Reference	Downregulated	Target gene(s)	Reference
Metabolism	miR-210	*ISCU1/2*	Liu et al. (2010a), Neal et al. (2010), Zhou et al. (2010)	miR-508-3p	*LDHA*	Zhou et al. (2010)
				miR-509-3p	*HK1*	
Cell Adhesion/invasion	NA			miR-141 and miR-200c	*ZEB2/ZFHX1B*	Nakada et al. (2008)
				miR-149	*LOX*	Liu et al. (2010a)
Apoptosis	miR-23b	*POX*	Liu et al. (2010b)	NA		
	miR-438-3p	*BBC3/PUMA*	Veronese et al. (2010)			
VHL-HIF pathway	miR-92a	*VHL*	Valera et al. (2011)	NA		
Angiogenesis	miR-29a	*TIS11B*	Sinha et al. (2009)	miR-200bc and miR-429	*VEGF*	Zhou et al. (2010)
Signal transduction	miR-34a	*SFRP1*	Liu et al. (2010a)	NA		
	miR-185	*PTPN13*				
	miR-224	*ERBB4*				
Other	miR-21	*SLC12A1, TCF21*	Liu et al. (2010a)	miR-141	*SEMA6A*	Liu et al. (2010a)
	miR-142-3p	*LRRC2*		miR-149	*KCNAB1, KCNMA1*	

BBC3/PUMA, BCL2 binding component 3; ERBB4, v-erb-a erythroblastic leukemia viral oncogene homolog 4; HK1, hexokinase 1; ISCU1/2, iron-sulfur cluster scaffold homolog 1/2; KCNAB1, potassium voltage-gated channel, shaker-related subfamily, beta member 1; KCNMA1, potassium large conductance calcium-activated channel, subfamily M, alpha member 1; LDHA, lactate dehydrogenase A; LOX, lysyl oxidase; LRRC2, leucine rich repeat containing 2; POX, proline dehydrogenase (oxidase) 1; PTPN13, protein tyrosine phosphatase, non-receptor type 13; VHL, Von Hippel–Lindau gene; SEMA6A, semaphorin 6A; SFRP1, secreted frizzled-related protein 1; SLC12A1, solute carrier family 12; TCF21, transcription factor 21; TIS11B, zinc finger protein 36; C3H type-like 1; VEGF, vascular endothelial growth factor; ZEB2/ZFHX1B, zinc finger E-box binding homeobox 2.

of IGF-I), and *KCNJ16* (a cell growth-related membrane protein). In addition, upregulation of miR-34a was correlated with downregulation of *SFRP1* (a Wnt signaling pathway regulator) whereas miR-224 upregulation was associated with downregulation of *ERBB4* (an EGFR family member and putative TSG; Liu et al., 2010a).

Hypoxic regulation of miRNAs is also emerging as an important mechanism implicated in RCC tumorigenesis (**Figure 1**). The deregulation of miRNAs in cell lines lacking VHL was been shown to be either mediated largely via HIF induction (miR-210 and miR-155) or by HIF independent VHL actions (miR-31, miR-21, miR-18a, miR-17, let-7i, miR-20a; Neal et al., 2010). Furthermore, the expression levels of HIF2α and of its downstream targets (VEGFα, TGFβ) seems to be regulated by several members of the miR-200 family (miR-141, miR-200a*, miR-200b, miR-200c), and downregulation of miR-200 (or miR-8) family and VHL loss activate the HIF pathway (Zhou et al., 2010). On the other hand, HIF1α can induce miR-210 in many solid tumors, and miR-210 has been consistently found to be overexpressed in ccRCC (Jung et al., 2009; Liu et al., 2010a; Neal et al., 2010; Zhou et al., 2010). Upregulation of miR-210 was associated with a reduced expression of its target gene *ISCU1/2*, which encodes assembly proteins involved in the biogenesis of [4Fe-4S] and [2Fe-2S] iron-sulfur clusters (Neal et al., 2010). These are implicated in electron transport and mitochondrial oxidation-reduction reactions, and, thus, downregulation of *ISCU1/2* might contribute to the repression of mitochondrial proteins and to the anaerobic metabolism in ccRCC (Neal et al., 2010). Interestingly, hypoxia induced microRNAs have been also correlated with *SLC12A1* and *TCF21* downregulation (encoding cell adhesion proteins) in ccRCC, whose mRNAs were identified as direct targets of hypoxia induced miR-21 (Liu et al., 2010a).

Only a few studies addressed the potential use of miRNAs as RCC biomarkers for detection. In this regard, differential miRNA expression patterns between neoplastic and non-neoplastic renal tissues, as well as among different renal tumor subtypes have been described. The discrimination between ccRCC and normal kidney tissue might be accomplished by a panel of nine miRs (miR-21, miR-34a, miR-142-3p, miR-155, miR-185, miR-200c, miR-210, miR-224, and miR-592; Juan et al., 2010), a combination of miR-141 and miR-155 (Jung et al., 2009) or through the differential expression of miR-92a, miR-210, and miR-200c (Valera et al., 2011).

Concerning distinctive miRNA signatures for each of the main RCT subtypes, unsupervised hierarchical cluster analysis of miRNA microarray data showed that tumors derived from the proximal nephron (ccRCC and pRCC type I) and tumors derived from the distal nephron (oncocytomas and chRCC) can be distinguished by their miRNA profile (Valera et al., 2011), extending previous observations for ccRCC and chRCC (Nakada et al., 2008). These differential expression patterns of microRNAs might be also used to subclassify RCT (Petillo et al., 2009; Fridman et al., 2010; Youssef et al., 2011). In ccRCC 23 miRNA are differentially expressed (let-7e, let-7f, let-7g, miR10b, miR-124, miR-126, miR-138, miR-140-5p, miR-142-5p, miR-144, miR-184, miR-200c, miR-203, miR-206, miR-210, miR-218, miR-27a, miR-27b, miR-335, miR-373, miR-378, miR-92a, miR-98; Valera et al., 2011). However, some miRNAs are characteristic of sporadic ccRCC (let-7c, let-7d, miR-1, miR-100, miR-10a, miR-148b,

Table 4 | Diagnostic and prognostic information in renal cell tumors provided by epigenetic biomarkers.

	Detection biomarkers	Reference	Prognostic/predictive biomarkers	Reference
DNA methylation	*APC/RARB2/RASSF1A*	Hoque et al. (2004)	*JUP*	Breault et al. (2005)
	RASSF1A	Peters et al. (2007)	*DAL1*	Yamada et al. (2006)
			APAF1/DAPK1	Christoph et al. (2006b)
	RASSF1A/PTGS2/CDH1	Costa et al. (2007)	*PTEN*	Kim et al. (2005)
Histone modifications & modifiers	NA		*H3K4me2/H3K18Ac*	Seligson et al. (2009)
			H3K4me1/H3K4me2/H3K4me3	Ellinger et al. (2010)
			H3K18Ac	Mosashvilli et al. (2010)
miRNA	miR-141/miR-155	Jung et al. (2009)	miR-32	Petillo et al. (2009)
	miR-1233	Wulfken et al. (2011)		

NA, not available.

miR-191, miR-199a-3p, miR-19a, miR-215, miR-29b, miR-30c, miR-363, miR-9) and others of hereditary (Von Hippel–Lindau syndrome-related) RCC (let-7a, miR-125a-5p, miR-125b, miR-143, miR-146b-5p, miR-15b, miR-17, miR-193a-5p, miR-193b, miR-196a, miR-20b, miR-214, miR-23b, miR-32, miR-372; Valera et al., 2011). Furthermore, ccRCC and pRCC differentially express 27 miRNAs, with miR-203 and miR-424 being more expressed in ccRCC than in pRCC, and miR-31 and mir-504 being more expressed in pRCC than in ccRCC (Petillo et al., 2009). Remarkably, a similar miRNA expression pattern was reported for chRCC and oncocytoma. These tumors share 60 miRNAs, with miR-143, miR-19a, miR-21, miR-29a, miR-181a, and miR-378 unique to chRCC and mir-146a being preferentially expressed in oncocytoma (Valera et al., 2011). Moreover, chRCC displayed higher levels of miR-203, mir-200b, miR-197, and miR-320 than oncocytoma, whereas miR-186 is more highly expressed in oncocytoma than in chRCC (Petillo et al., 2009). Finally, a recent study which investigated miRNAs expression levels in sera of RCC patients and healthy controls, identified miR-1233 as promising biomarker for RCC detection and monitoring (Wulfken et al., 2011).

Altered levels of miRNA might also provide prognostic information. Whereas miR-155 and miR-21 expression in ccRCC tumors has been found to correlate with tumor size (Neal et al., 2010), higher miR-210 levels were found in tumors displaying higher Fuhrman grade (Valera et al., 2011). In addition, in ccRCC, overexpression of miR-32 as well as of miR-210, miR-21, let-7i, and miR-18a correlated with poor survival (Petillo et al., 2009; Neal et al., 2010) and lower miR-106b levels were associated with metastatic disease and poorer relapse-free survival (Slaby et al., 2010). High miR-210 expression was also found in tumors with lymph node metastasis (Valera et al., 2011), suggesting unique miRNA signatures in RCC metastasis, distinct from those of primary tumors (White et al., 2011).

CONCLUSION

The ubiquity of epigenetic alterations in RCT supports their fundamental role in renal carcinogenesis. Those alterations not only provide further insight into the complex mechanisms underlying the genesis and progression of RCT, but they also grant the opportunity for the development of innovative biomarkers which might aid in disease detection, diagnosis, assessment of prognosis, and prediction of response to therapy (**Table 4**). Finally, owing to the reversible and plastic nature of epigenetic alterations, these constitute an attractive target for novel therapeutic approaches that might tackle one of the most chemoresistant types of human cancer.

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CHAPTER 2

RATIONALE AND AIMS

Epigenetic alterations might provide clinically useful biomarkers for accurate detection of renal cell tumous (RCT), distinction among different RCT and assessment of prognosis, through gene promoter hypermethylation, histone modifying enzymes and miRNA expression analyses. These alterations might be used in a panel to distinguish among RCT subtypes, but validation in clinical series is still lacking, precluding its translation to the clinical setting. Additionally, different patterns of histone modifications and histone modifying enzymes among RCT types have not been fully explored.

General aim

The main aim of this thesis is to assess the clinical usefulness of epigenetic-based biomarkers in renal cell tumors, especially their diagnostic and prognostic value.

We also expect that the data which derived from this project will contribute for the elucidation of epigenetic mechanisms involved in renal carcinogenesis, as well as for the implementation of new early detection, personalized treatment and personalized follow-up strategies in renal cell tumors.

Specific aims

- Systematize the patterns of epigenetic alterations, focusing on promoter hypermethylation and histone oncomodifications/modifying enzymes, of the

four most frequent renal cell tumors: clear cell, papillary and chromophobe renal cell carcinomas, and oncocytoma.

- Define a biomarker panel allowing for accurate diagnosis of renal cell tumors.
- Assess the prognostic value of promoter methylation and histone modifying enzymes expression in renal cell carcinoma.

CHAPTER 3

METHODS

Given that each paper has its own Methods sections, and to avoid unnecessary repetition, only a brief overview of the techniques performed by the candidate will be enumerated in this chapter.

Patients and sample collection

- Fresh-frozen tissue of nephrectomy specimens (tumor and normal), collected since 2000 to 2015, was used for this project.
- Cases were selected after pathological review, and relevant information was collected from clinical files:
    - Promoter methylation analysis – 30 clear cell renal cell carcinoma, 30 papillary renal cell carcinoma, 30 chromophobe renal cell carcinoma, 30 oncocytoma, in a total of 120 cases; 10 morphologically normal renal tissue samples from nephrectomy specimens due to urothelial carcinoma;
    - Histone modifying enzymes – cohort #1: additional 10 cases for each renal cell tumor histotype, in a total of 160 cases; 10 morphological normal renal tissue from nephrectomy specimens due to urothelial carcinoma;
    - Histone modifying enzymes – cohort #2: 62 clear cell renal cell carcinoma, 31 with metastasis during follow-up and 31 without metastasis.

- This study was approved by the Institutional Review Board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute – Porto, Portugal (CES518/2010).

Cell lines and demethylating treatment

- Culture of clear cell renal cell carcinoma cell lines – 769-P, 786-O, Caki-1 [from the American Type Culture Collection (Manassas, VA)];
- Treatment with the demethylating drug 5-aza-2'deoxycytidine for 72h.

Nucleic acid extraction and cDNA synthesis

- DNA extraction from fresh-frozen tissue and cell lines [phenolchloroform];
- RNA extraction from fresh-frozen tissue and cell lines [TRIzol^{R} reagent (Invitrogen^{TM}, Carlsbad, CA, USA)];
- cDNA synthesis [TransPlex^{R} Whole Transcriptome Amplification Kit (Sigma-Aldrich^{R}, St. Louis, MO, USA) and High Capacity cDNA Reverse Transcription kit (Applied Biosystems^{R}, Foster City, CA, USA)].

Methylation analysis

- Screening of promoter methylation [EpiTect Methyl II qPCR array (SABiosciences, Qiagen, Frederick, MD, USA)]
- Bisulfite treatment [EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA)]

- Primer design [Methyl Primer Express v 1.0 software (Applied Biosystems, Foster City, CA, USA)]
- Quantitative methylation specific polymerase chain reaction (QMSP) [SYBR^{R} Green technology]

Expression analysis

- Evaluation of mRNA expression level, after cDNA synthesis, by quantitative polymerase chain reaction using custom-made primers and commercial expression assays.

Immunohistochemical analysis

- Evaluation of protein expression by immunohistochemistry, using a semi-quantitative scale for staining intensity and percentage of positive cells.

CHAPTER 4

METHYLATION IN RENAL CELL TUMORS

4.1. CHAPTER OVERVIEW

The results presented in this chapter are published in international peer reviewed journals:

- ***Pires-Luís AS**, Costa-Pinheiro P, Ferreira MJ, Antunes L, Lobo F, Oliveira J, Henrique R, Jerónimo C. Identification of clear cell renal cell carcinoma and oncocytoma using a three-gene promoter methylation panel. J Transl Med, 2017; 15 (1): 149*;
- ***Pires-Luís AS**, Vieira-Coimbra M, Ferreira MJ, Ramalho-Carvalho J, Costa-Pinheiro P, Antunes L, Dias PC, Lobo F, Oliveira J, Graça I, Henrique R, Jerónimo C. Prognostic significance of MST1R dysregulation in renal cell tumors. Am J Cancer Res, 2016; 6(8):1799-1811.*

RATIONALE

Promoter methylation has emerged as a promising biomarker class in urologic cancers, specifically in kidney tumors [1, 2]. Despite distinct patterns of methylations have been described for clear cell, papillary and chromophobe carcinoma, and also for oncocytoma [3, 4], validation in independent series is scarce and precludes its assessment as useful tools for clinical practice.

Additionally, the functional implications of promoter methylation of some genes known to be methylated in renal cell carcinoma might be relevant for tumor aggressiveness and prognosis [5].

MAJOR FINDINGS

- A panel comprising the promoter methylation level of *OXR1* and *MST1R* is a highly sensitive and specific diagnostic biomarker for renal tumors (98% sensitivity, 100% specificity) and for clear cell renal cell carcinoma (90% sensitivity, 98% specificity).
- *OXR1* promoter methylation level was significantly higher in high grade clear cell renal cell carcinoma.
- *MST1R* promoter methylation was associated with transcription regulation in renal cell tumors, and MST1R expression was associated with prognosis.

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4.2. IDENTIFICATION OF CLEAR CELL RENAL CELL CARCINOMA AND ONCOCYTOMA USING A THREE-GENE PROMOTER METHYLATION PANEL

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RESEARCH Open Access

Identification of clear cell renal cell carcinoma and oncocytoma using a three-gene promoter methylation panel

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Abstract

Background: Promoter methylation has emerged as a promising class of epigenetic biomarkers for diagnosis and prognosis of renal cell tumors (RCTs). Although differential gene promoter methylation patterns have been reported for the major subtypes (clear cell, papillary and chromophobe renal cell carcinoma, and oncocytoma), validation of diagnostic performance in independent series have been seldom performed. Herein, we aimed at assessing the diagnostic performance of genes previously shown to be hypermethylated in RCTs in different clinical settings.

Methods: Promoter methylation levels of *HOXA9* and *OXR1* were assessed by quantitative methylation specific PCR. ROC curves were generated for *OXR1*, *OXR1* combined with *MST1R* and *HOXA9*. Sensitivity, specificity, positive predictive value, negative predictive value and accuracy were computed, maximizing specificity. Methylation levels were also correlated with clinical and pathological relevant parameters.

Results: *HOXA9* and *OXR1* promoter methylation was disclosed in 73 and 87% of RCTs, respectively. A two-gene methylation panel comprising *OXR1* and *MST1R* identified malignancy with 98% sensitivity and 100% specificity, and clear cell renal cell carcinoma with 90% sensitivity and 98% specificity. *HOXA9* promoter methylation allowed for discrimination between oncocytoma and both papillary and chromophobe renal cell carcinoma but only with 77% sensitivity and 73% specificity. Significantly higher OXR1 promoter methylation levels ($p = 0.005$) were associated with high nuclear grade in ccRCC.

Conclusions: A panel including *OXR1* and *MST1R* promoter methylation allows specific and sensitive identification of renal cell tumors, and, especially, of clear cell renal cell carcinoma. Moreover, higher *OXR1* promoter methylation levels associate with clear cell renal cell carcinoma nuclear grade, a surrogate for tumor aggressiveness. Thus, gene promoter methylation analysis might a useful ancillary tool in diagnostic management of renal masses.

Keywords: Kidney tumours, Renal cell tumour, Clear cell renal cell carcinoma, Oncocytoma, Epigenetics, Methylation, *OXR1*, *HOXA9*, *MST1R*, Diagnostic biomarker

Background

Epigenetic deregulation is a frequent finding in renal cell tumors (RCT) [1]. These arise from renal cortical tubular cells and encompass several entities, the most frequent being clear cell renal cell carcinoma (ccRCC), papillary renal cell carcinoma (pRCC) and chromophobe renal cell carcinoma (chRCC), representing 75, 10–15 and 5% of all RCT respectively. These are malignant neoplasm, although of variable aggressiveness. Indeed, ccRCC and pRCC are those that most frequently progress through regional and systemic metastization, whereas chRCC is generally more indolent. Among benign tumors, the most frequent RCT is oncocytoma [2].

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The differential diagnosis among specific RCT subtypes can be challenging, especially in those tumors composed of cells with granular eosinophilic cytoplasm, as some morphological and immunohistochemical overlap exists among oncocytoma, eosinophilic variant of chRCC and eosinophilic variant of ccRCC [2, 3]. However, since their prognosis is radically different, accurate discrimination among these entities is critical. Furthermore, RCT therapy is becoming progressively more conservative, especially those of small size, with increasing use of cryoablation or radiofrequency techniques [4], entailing the need for more accurate diagnosis in biopsy samples. Owing to the heterogeneity that characterizes RCTs, a small tumor tissue sample might impair an accurate diagnosis based on histopathological, histochemical and immunohistochemical features [4, 5]. In this context, epigenetic biomarkers may constitute a valuable ancillary tool for diagnosis in biopsies from renal masses.

Among epigenetic alterations, aberrant promoter methylation, which generally entails gene silencing [6], has emerged as a promising class of biomarkers in urologic neoplasms [7], including RCTs [8, 9]. Although several genes are known to be hypermethylated in RCTs, mostly in ccRCC [10], frequencies vary, with most genes displaying intermediate (20–70%) methylation frequencies. Among genes with consistently high (>70%) methylation frequency in RCC, *APAF1* [11, 12], *MDR1* [13], and *PTGS2* [13], should be highlighted (97–100, 86 and 94%, respectively). Recently, we showed that *MST1R* was also frequently methylated in RCC, and promoter methylation levels discriminated ccRCC from the remaining RCT subtypes with high specificity [14].

Nevertheless, over the last years, several high-throughput studies on RCC promoter methylation using an array-based approach, identified several other hypermethylated genes in RCC, which might be useful as diagnostic biomarkers. This somewhat extensive list includes *SPINT2* [15], *IGFBP1*, *IGFBP3*, *COL1A1* [16], *UCHL1* [17], *CXCL16*, *KTN19* [18], *IGFBP2*, *SOX17*, *COL1A2*, *BMP4*, *FRZB*, *TAL1*, *MCM2*, *KCNK4*, *HOXC6*, *CCNA1*, *HOXA11*, *TERT*, *TMEFF2*, *PGF*, *ZNF215*, *SMARCB1*, *TWIST1*, *IGFBP7* [19], *BNC1*, *COL14A1*, *SFRP1* [20], *PCDH8*, *CCDC8*, and *FBN2* [21]. Most of these studies, however, mostly focused on ccRCC, only, whereas other included the most frequent RCTs and identified specific methylation patterns for each subtype in general [22], or specifically for the distinction between chRCC and oncocytoma [23]. Still, diagnostic performance analysis of these putative RCT biomarkers has not been performed. Thus, we aimed to evaluate the diagnostic performance of promoter methylation of several genes previously identified as candidate RCT biomarkers in array studies [22, 23], including also *MST1R* [14], in several differential diagnosis scenarios.

Methods

Patients, sample collection and DNA extraction

Representative tumor tissue was collected from 120 patients, submitted to radical or partial nephrectomy at the Portuguese Oncology Institute of Porto (Portugal) between 2003 and 2007, comprising ccRCC, pRCC, chRCC and oncocytoma (30 cases of each). Additionally, morphologically normal kidney (cortical) tissue from 9 nephrectomy specimens for upper urinary tract neoplasia were also collected and served as controls.

Tissue samples were snap-frozen immediately after surgery, stored at −80 °C and subsequently cut in a cryostat. The presence of at least 70% of tumor cells in the sections was assessed in H&E stains. Genomic DNA extraction was performed as previously described [24]. Briefly, 10% SDS was added to the sample, then proteinase K (20 mg/mL, overnight, 55 °C) to digest DNA, followed by extraction with phenol–chloroform and precipitation with 100% ethanol.

Formalin-fixed paraffin-embedded routine sections were used for routine tumour classification and grading (WHO) as well as staging (TNM) [2]. Relevant clinical data was retrieved from clinical charts.

This study was approved by the Institutional Review Board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute of Porto, Portugal (CES518/2010).

Gene selection

MST1R (GenBank: NM_002447) promoter methylation was previously identified by our group through EpiTect Methyl II qPCR array (SABiosciences, Qiagen, Frederick, MD, USA), and proved to be a specific diagnostic biomarker for ccRCC [14]. Briefly, 20 samples (4 ccRCC; 4 pRCC; 6 chRCC; 6 oncocytoma) were tested with the EMT commercial assay (Cat. No. 524EAHS-901ZA-24) on a 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to manufacturer instructions. *MST1R*, the gene with the highest percent of hypermethylated DNA (representing the fraction of input DNA containing at least two methylated CpG sites in the targeted region) was selected for further analysis, and proved to be a specific ccRCC biomarker [14].

Relevant literature was also reviewed, focusing on methylation array studies comparing different RCT subtypes, and two additional genes were selected: *OXR1* (GenBank: NM_001198532.1), proposed as a promising diagnostic biomarker for proximal tubule-derived RCC (ccRCC and pRCC) [22]; and *HOXA9* (GenBank: NM_152739.3) owing to its potential to distinguish chRCC from RO [23].

Bisulfite treatment

Bisulfite treatment to convert unmethylated cytosine to uracil, maintaining methylated cytosine as such, was performed with EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions, in 169 fresh-frozen samples: 9 morphologically normal kidneys, 30 ccRCC, 30 pRCC, 30 chRCC, 30 RO, 20 bladder urothelial carcinoma and 20 prostate adenocarcinoma samples.

Quantitative MSP—fresh-frozen tissues

Primers for the candidate genes were designed to amplify methylated bisulfite converted complementary sequences, using Methyl Primer Express v 1.0 (Applied Biosystems, Foster City, CA, USA), considering the best predicted primer pair for the promoter region of each gene. Primers are listed in Table 1. A reference gene (β-*actin*) was used to normalize for DNA input in each sample.

For fresh-frozen tissues, quantitative real-time polymerase chain reaction (qMSP) was performed in an 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA), after DNA bisulfite treatment, in a reaction volume of 20 μL consisting of 10 μL of SYBR ® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 7 μL of H_2O, 0.5 μL of forward primer, 0.5 μL of reverse primer and 2 μL of bisulfate-modified DNA. Each sample was run in triplicate. In each plate, "no template controls" were included as a control for contamination, and a calibration curve was constructed with serial dilutions (1:5) of bisulfite converted universally methylated DNA at all CpGs (CpGenome Universal Methylated DNA; Millipore, Billerica, MA), to quantify the amount of fully methylated alleles in each reaction. The amplification reaction was carried out at 95 °C for 2 min, then 45 cycles of 95 °C for 15 s and annealing temperature (60 °C for all genes) for 1 min, followed by melting curve analysis.

For each sample, the relative level of methylated promoter DNA was determined by the ratio between the mean quantity obtained by qMSP analysis for each gene and the mean quantity of the internal reference gene (*ACTB*), multiplied by 1000 for easy tabulation, according to the formula: methylation level = (target gene/reference gene) × 1000.

Table 1 Primer sequences used in quantitative methylation specific PCR for candidate genes

Primer set	Sense primer sequence (5′–3′)	Antisense primer sequence (5′–3′)
MST1R[a]	AGCGTTAGTGTATAGCGGC	TAAACAACGATCCCGACA
OXR1	TTCGTTGTATATATCGAACGGT	CCGTACTAAATATCTCGTTAACT
HOXA9	TATTTAGTCGGTATTCGC	ACCTCGAACGCTTCCAT

[a] *MST1R* promoter methylation primers from [14, 25]

Statistical analysis

The frequency of methylated samples was determined for each RCT type, considering the highest value determined in the normal kidney tissue as cutoff. Median and interquartile range of methylation levels were also computed. Kruskal–Wallis non-parametric ANOVA followed by Mann–Whitney U test (with Bonferroni's correction) for pair-wise comparisons were used to identify significant differences in methylation levels among RCT subtypes and association with standard clinicopathological variables. Spearman's test was performed to ascertain correlation between age and methylation levels.

Methylation levels of *OXR1* and *MST1R* were combined using a logistical regression model by computing a new variable with the predicted values.

To assess the performance of promoter methylation levels as diagnostic biomarkers, receiver operator characteristics (ROC) curves were constructed by plotting the true positive rate (sensitivity) against the false positivity rate (1-specificity), followed by computation of the area under the curve (AUC). Cutoff values based on ROC curve analysis, prioritizing specificity and then sensitivity, were selected for calculation of sensitivity, specificity, positive and negative predictive values, and accuracy.

Disease specific survival (time between diagnosis and death for renal cell carcinoma), disease free survival (time between treatment and the first metastasis or local recurrence) and overall survival (time between diagnosis and death irrespective of cause) curves were constructed using the Kaplan–Meier method, with log-rank test (univariable analysis) and Cox regression analysis, for standard clinicopathological variables (age, gender, histological subtype, pathological stage) and methylation level of candidate genes. For this purpose, candidate gene methylation levels were classified as low or high using the 75th percentile methylation value of each gene as cutoff, and age, stage and histological subtype were dichotomized as age <75 vs ≥75, stage I and II vs stage III and IV and pRCC vs ccRCC.

Statistical significance level was set at $p < 0.05$ (two-sided). Analysis was performed using IBM ® SPSS ® Statistics for Windows, version 22.0 (SPSS, Chicago, IL, USA). Graphs were built using GraphPad Prism 6.0 software for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

Promoter methylation analysis by qMSP

Tumor samples were categorized as *HOXA9* or *OXR1* methylated using the respective highest methylation ratio value observed in normal/control samples as cutoff (14.11 for *HOXA9* and 1577.45 for *OXR1*). Using these criteria, 73 and 87% of tumor samples were considered

hypermethylated at *HOXA9* and *OXR1* promoters, respectively. Considering each subtype, the highest *HOXA9* promoter methylation frequency was found in oncocytomas (93%), followed by ccRCC (70%), chRCC (67%) and pRCC (60%), whereas the highest *OXR1* promoter methylation frequency was found in pRCC (93%), followed by ccRCC and oncocytoma (87%), and then chRCC (80%).

Levels in RCTs were significantly higher than in normal kidney ($p < 0.001$) (Fig. 1a). Moreover, *HOXA9* and *OXR1* promoter methylation also differed between benign (RO) and malignant (RCC) renal cell tumors ($p = 0.011$ and $p = 0.009$, respectively) (Fig. 1b). Among RCT subtypes, *OXR1* methylation were significantly higher in ccRCC compared to the remaining three subtypes ($p < 0.001$ for all), and also in pRCC compared to chRCC ($p < 0.001$) (Fig. 1c2). Concerning *HOXA9*, promoter methylation levels were only significantly higher in RO compared to chRCC ($p = 0.004$) (Fig. 1c1).

Diagnostic performance of candidate biomarkers

Both high *HOXA9* and *OXR1* promoter methylation levels discriminated normal from tumour samples with good sensitivity and high specificity (73 and 89% for *HOXA9* and 87 and 100% for *OXR1*, respectively) (Table 2). High *OXR1* promoter methylation levels also discriminated ccRCC from the remaining RCTs tested (pRCC, chRCC and RO) with 80% sensitivity and 93% specificity (AUC = 0.847) (Table 2; Fig. 2a).

Considering these results and those that we previously reported for *MST1R* [14], a gene panel combining *OXR1* and *MST1R* gene promoter methylation was tested, and diagnostic performance increased for discrimination between ccRCC vs RCTs, displaying 90% sensitivity and 98% specificity (AUC = 0.939) (Table 2; Fig. 2b). Then, using *HOXA9* promoter methylation levels, RO could be discriminated from pRCC and chRCC with 77% sensitivity and 73% specificity (Table 2; Fig. 2c). A proposed combined use of these biomarkers is depicted in Fig. 3.

Clinicopathological correlates

Relevant clinical and pathological data of the 120 RCT patients included in this study are depicted in Table 3 [14, 25]. The 9 patients from which normal kidney tissue was retrieved presented a median age of 69 years (range: 20–83), and 6 (67%) were males. No statistically significantly differences between RCT and normal kidney samples were found for age ($p = 0.24$) nor gender (0.453).

OXR1 and *HOXA9* promoter methylation levels did not correlate with age ($p = 0.08$ and $p = 0.18$, respectively)

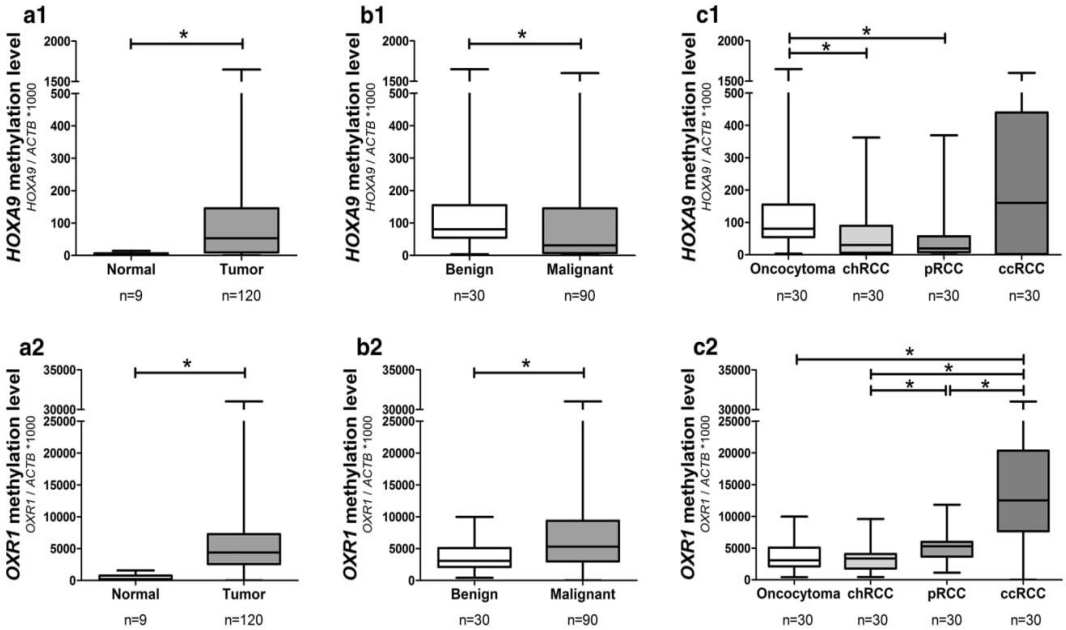

Fig. 1 *OXR1* and *HOXA9* promoter methylation levels. Methylation levels in 9 normal renal tissue and 120 RCT samples for *HOXA9* (**a1**) and *OXR1* (**a2**); in 30 benign (oncocytoma) and 90 malignant (renal cell carcinoma) for *HOXA9* (**b1**) and *OXR1* (**b2**); and in the four RCT subtypes *HOXA9* (**c1**) and *OXR1* (**c2**). Target gene methylation level = target gene mean quantity/ACTB mean quantity * 1000). *chRCC* chromophobe renal cell carcinoma, *pRCC* papillary renal cell carcinoma, *ccRCC* clear cell renal cell carcinoma, *RCT* renal cell tumour

Table 2 Diagnostic performance of *OXR1*, *OXR1&MST1R*, and *HOXA9* promoter methylation in different clinical settings

	SE (%)	SP (%)	PPV (%)	NPV (%)	Accuracy (%)
Normal vs tumour					
OXR1 methylation	87	100	100	36	88
OXR1&MST1R methylation	98	100	100	75	98
HOXA9 methylation	73	89	99	20	74
ccRCC vs RCT					
OXR1 methylation	80	93	80	93	90
OXR1&MST1R methylation	90	98	93	97	96
RO vs pRCC&chRCC					
HOXA9 methylation	20	95	67	70	70

SE sensitivity, *SP* specificity, *PPV* positive predictive value, *NPV* negative predictive value, *RCT* renal cell tumour, *ccRCC* clear cell renal cell carcinoma, *pRCC* papillary renal cell carcinoma, *chRCC* chromophobe renal cell carcinoma, *RO* renal oncocytoma

or gender (p = 0.46 and p = 0.15, respectively). Considering all RCCs, methylation levels of *OXR1* and *HOXA9* were not associated with stage (p = 0.143 and p = 0.254 respectively) nor with the development of metastasis (p = 0.055 and p = 0.467 respectively). In ccRCC and pRCC, *OXR* (p = 0.008), but not *HOXA9*, promoter methylation levels associated with nuclear grade. Considering each histological subtype separately, higher *OXR1* promoter methylation levels were observed in high (3 and 4) grade tumours (median: 16,714; interquartile range: 11,993–21,817) compared to low (1 and 2) grade tumours (median: 7300; interquartile range: 355–10,715) in ccRCC only (p = 0.005) (Fig. 4).

No significant differences were found for age, gender or stage in ccRCCs that presented higher vs lower *OXR1* methylation levels (cutoff = median value of *OXR1* promoter methylation levels distribution, p = 0.486, p = 0.700 and p = 0.109, respectively).

Survival analysis

During follow-up [median (range): 60 months (2–392 months)], 12 (13%) patients died from RCC and 17 (19%) developed metastatic disease.

Stages III and IV were associated with shorter cancer specific survival [HR: 13.5 (3–62), p = 0.001], disease-free survival [HR: 4.5 (1.7–12), p = 0.002] and overall survival [HR: 2.8 (1.3–6.1), p = 0.01] when compared to stages I and II, as expected. Considering only ccRCC and pRCC, the subtypes that most frequently display metastatic spread, pRCC was associated with shorter overall survival [HR: 2.7 (1.1–6.6), p = 0.033].

Higher *OXR1* or *HOXA9* promoter methylation levels were not associated with worse disease specific, disease free or overall survival.

Discussion

Renal cell tumours, the most frequent (85–90%) kidney tumours, were classically diagnosed in advanced stage (IV), with large size, presence of metastasis and dismal prognosis when compared to kidney-confined tumours, which can usually be cured by complete surgical resection. However, with the increasing number of abdominal imaging studies performed due to unrelated symptoms, the number of incidentally diagnosed tumours has increased, posing new clinical challenges. These incidental tumours tend to be smaller (<5 cm) and kidney-confined, allowing complete surgical resection by partial nephrectomy in a high proportion of cases, or even alternative therapeutic strategies, including cryoablation or

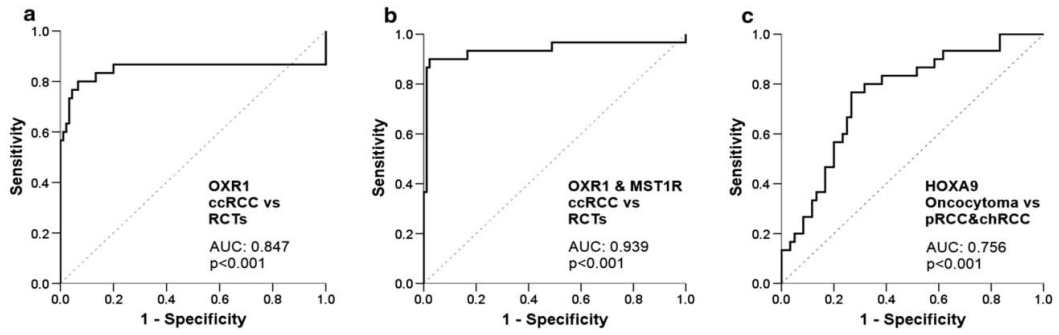

Fig. 2 ROC curves for *OXR1*, *OXR1&MST1R* and *HOXA9* promoter methylation levels in different clinical settings. ROC curves for clear cell renal cell carcinoma (ccRCC) versus the remaining renal cell tumours (RCT) for *OXR1* (**a**) and for *OXR1&MST1R* (**b**), and for oncocytoma versus papillary (pRCC) and chromophobe (chRCC) renal cell carcinoma for *HOXA9* (**c**)

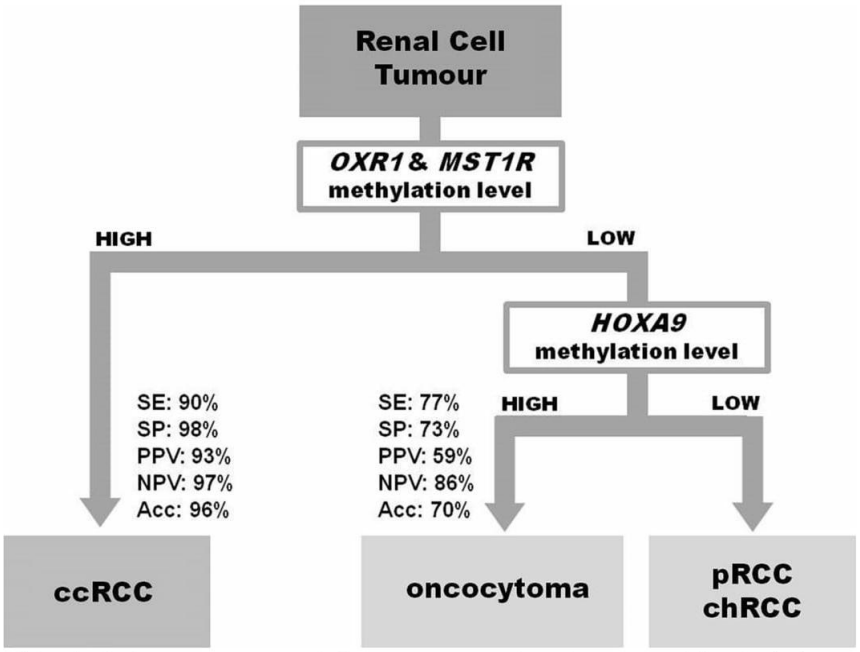

Fig. 3 Proposed algorithm for discriminating among renal cell tumours in tissue samples. High promoter methylation levels of OXR1 combined with MST1R allowed identification of renal cell tumours (RCT) from normal renal tissue, and of clear cell renal cell carcinoma (ccRCC) from the remaining renal cell tumours (RCT). Then, a high promoter methylation level of HOXA9 allowed the identification of oncocytoma from papillary (pRCC) and chromophobe (chRCC) renal cell carcinoma. *SE* sensitivity, *SP* specificity, *PPV* positive predictive value, *NPV* negative predictive value, *Acc* accuracy

radiofrequency ablation. In these cases, renal mass biopsy is mandatory, for adequate risk stratification, which requires accurate diagnosis [4, 26]. However, despite tumour subtype identification being globally accurate (>90%) in renal mass biopsy, it is non-diagnostic in approximately 15% of patients, more frequently in small renal tumours. Moreover, it could underestimate tumour grade and stage in 25 and 5–10% of patients, respectively, and fail identification of pathologic features associated with aggressiveness (e.g., sarcomatoid differentiation) mostly due to sampling limitations [27]. In this context, diagnostic epigenetic biomarkers, including promoter methylation, might be clinically useful, mainly in patients considered for ablative techniques, in which renal mass biopsy is the sole available source of tumor material [27, 28]. Although several genes were consistently reported to be hypermethylated in RCC, and methylation array based studies reported different methylation patterns in distinct RCT subtypes [22, 23], validation in independent series has been seldom performed.

For this study, we selected three genes—*OXR1*, methylated in ccRCC and pRCC [22]; *HOXA9*, reported as differentially methylated in chRCC and oncocytomas [23]; and *MST1R*, highly methylated in ccRCC, that we previously shown to accurately identify ccRCC [14]—to assess their diagnostic performance in an independent series of 120 RCTs. Using robust methylation-specific primers for each gene promoter and performing quantitative methylation-specific PCR, we found that *OXR1* and *MST1R* promoter methylation discriminated between normal renal tissue and renal cell tumours with high specificity. Moreover, higher *OXR1* and *MST1R* methylation levels were characteristic of ccRCC (90% sensitivity and 98% specificity). Thus, this biomarker panel might be useful as ancillary diagnostic tool in renal mass biopsies with ambiguous morphological findings or limited tissue for microscopic evaluation. Furthermore, in patients with low *OXR1* and *MST1R* methylation level (NPV: 97%), *HOXA9* methylation level distinguished oncocytoma from chRCC and pRCC. This biomarker might be useful in cases in which Hale's colloidal iron and immunohistochemistry (CK7, CD15) do not allow for a confident differential diagnosis between oncocytoma and chRCC [5]. This information might be clinically useful, not only

Table 3 Clinical and pathological features of the 120 RCT patients included in the study

	Tumour
Number of patients, n	120
Age, median (range)	60 (29–83)
Gender, n (%)	
Male	73 (61)
Female	47 (39)
Histological subtype, n (%)	
Clear cell RCC	30 (25)
Papillary RCC	30 (25)
Chromophobe RCC	30 (25)
Oncocytoma	30 (25)
Pathological stage, n (%)	
Stage I	47 (39)
Stage II	19 (16)
Stage III	21 (17.5)
Stage IV	3 (2.5)
n.a. (oncocytoma)	30 (25)
Nuclear grade, n (%)	
Grade 1	3 (2.5)
Grade 2	23 (19)
Grade 3	29 (24.5)
Grade 4	5 (4)
n.a. (chRCC and oncocytoma)	60 (50)
Metastasis during follow-up	
Clear cell RCC	9 (7.5)
Papillary RCC	7 (5.8)
Chromophobe RCC	1 (0.8)

RCC renal cell carcinoma, *chRCC* chromophobe renal cell carcinoma, *n.a.* not applicable

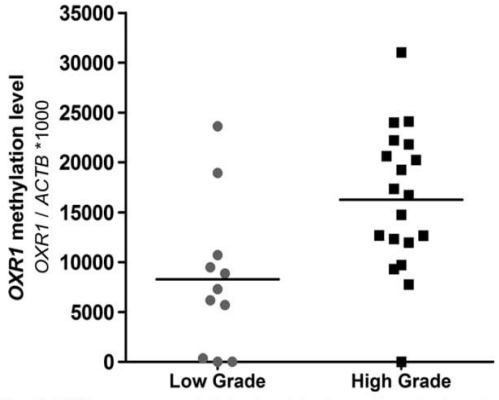

Fig. 4 *OXR1* promoter methylation levels in clear cell renal cell carcinoma (ccRCC) according to nuclear grade. High grade (grade 3 and grade 4) clear cell renal cell carcinomas display higher *OXR1* promoter methylation level than low grade (grade 1 and grade 2) tumours ($p = 0.005$)

to decide the best therapeutic strategy but also to select patients for active surveillance protocols [26, 27].

These results compare well with the reported performance of other DNA methylation-based biomarkers. *PCDH17* and *TCF21* promoter methylation identified renal cell tumours with 67% sensitivity and 100% specificity [29], but *OXR1* and *MST1R* were equally specific (100%) but more sensitive (98%) in the distinction between RCT and normal renal tissue. *PTGS2* was reported to distinguish ccRCC from the remaining RCTs subtypes with 46% sensitivity and 91% specificity [13], and we demonstrated that *OXR1* and *MST1R* reached a superior performance in all validity estimates. Moreover, *RASSF1A* hypermethylation was shown to discriminate pRCC from normal renal tissue with 87.5% sensitivity and 73.3% specificity, although comparison with other RCT subtypes was not undertaken [30]. Recently, an Illumina Infinium HumanMethylation450 (HM450) DNA methylation model for subtype prediction (encompassing angiomyolipoma, oncocytoma, ccRCC, pRCC and chRCC) that includes 59 variables (2 for angiomylolipoma, 9 for oncocytoma, 11 for normal kidney, 13 for ccRCC, 14 for pRCC and 10 for chRCC) was reported [31]. This model predicted for malignancy in 93% of samples, the correct subtype in 85% of RCT samples and 91% of ccRCC in the validation cohort (272 ex vivo core biopsies) [31]. Although we used a simpler and less expensive approach, correct ccRCC identification was reached in 98% of samples with the two-gene panel. Several studies focused on detection of aberrant promoter methylation in urine samples for RCT diagnosis. Nonetheless, the sensitivity was significantly lower than in tissue samples [29, 32, 33], and additional technical developments are warranted. Other epigenetic biomarker panels allowing for discrimination among RCT subtypes have been reported. A microRNA panel comprising miR-141 and miR-200b identified RCTs with high specificity and sensitivity (100 and 99%, respectively), discriminating oncocytoma from RCC and from chRCC with 86 and 90% sensitivity, respectively [34]. This performance is similar to that of *OXR1* and *MST1R* methylation panel for RCT (98% sensitivity, 100% specificity), although this panel did not perform as well concerning oncocytoma vs RCC in general.

Interestingly, some associations between promoter methylation levels and clinicopathological parameters were disclosed, although no impact in patient survival was apparent, probably due to the low number of events during follow-up. Indeed, high grade ccRCC displayed higher *OXR1* methylation levels than low grade ccRCC. This might be of clinical relevance as the most recently published biopsy series reveal high accuracy for RCT subtype identification, but poor reproducibility

for tumour grading [4, 27]. Tumour grade is an important criterion for risk stratification of small renal masses, contributing for decisions about clinical management, i.e., either recruitment for active surveillance protocols, or selection for nephrectomy or ablative therapies [35]. Hence, a diagnostic biomarker that, in addition to histological subtype, also conveys information about tumor aggressiveness might improve risk stratification algorithms in biopsies from small renal masses.

The main limitations of this study concerns to the use of fresh-frozen tissue from renal tumours for molecular analysis, requiring validation in formalin-fixed paraffin embedded tissues from biopsy specimens before clinical implementation. Furthermore, the sensitivity and specificity for identification of oncocytoma requires improvement, eventually through the addition of another marker. Finally, the limited number of kidney cancer-related deaths and progression events impaired survival analysis.

Conclusions

A panel including *OXR, MST1R* and *HOXA9* promoter methylation might be useful for positive identification of RCT, as well as for discrimination among subtypes. This panel could be used as ancillary diagnostic tool in the setting of renal mass biopsy, in which the amount of tissue available for histopathological examination may preclude a definitive diagnosis. Moreover, the panel might also improve risk stratification of patients harboring small renal masses, assisting clinicians in defining the best therapeutic strategy. Nevertheless, validation in larger independent cohorts is warranted to confirm the clinical potential of this gene methylation panel.

Abbreviations

RCT: renal cell tumour; RCC: renal cell carcinoma; ccRCC: clear cell renal cell carcinoma; pRCC: papillary renal cell carcinoma; chRCC: chromophobe renal cell carcinoma; RO: renal oncocytoma; OXR1: oxidation resistance gene 1; HOXA9: homeobox A9; MST1R: macrophage stimulating 1 receptor; ROC: receiver operating characteristic; AUC: area under the curve; SE: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value; CNV: copy number variation.

Authors' contributions

Conceived and designed the experiments: ASPL, RH, CJ. Performed the experiments: ASPL, PCP and MJF. Data analysis and interpretation: ASPL, LA, RH and CJ. Contributed reagents/material/analysis: FL, JO, RH, CJ. Manuscript preparation: ASPL, RH and CJ. All authors read and approved the final manuscript.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute-Porto, Portugal (CES518/2010).

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4.3. PROGNOSTIC SIGNIFICANCE OF MST1R DYSREGULATION IN RENAL CELL TUMORS

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Original Article

Prognostic significance of *MST1R* dysregulation in renal cell tumors

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Abstract: Macrophage stimulating 1 receptor (*MST1R*) is a C-MET proto-oncogene family receptor tyrosine kinase. Promoter methylation patterns determine transcription of *MST1R* variants as hypermethylation of a region upstream of transcription start site (TSS) is associated with lack of MST1R long transcript ($MST1R_{long}$) and expression of a short transcript with oncogenic potential. Thus, we aimed to investigate *MST1R* variant transcript regulation in renal cell tumors (RCT) and assess their prognostic potential. We found, in a series of 120 RCT comprising the four main subtypes (clear cell, papillary and chromophobe renal cell carcinoma, and oncocytoma), that higher methylation levels close to TSS were associated with total *MST1R* expression levels ($MST1R_{total}$) in primary tumors (p=0.049) and renal cancer cell lines. After demethylating treatment, $MST1R_{long}/MST1R_{total}$ ratio increased, as expected, in two renal cell carcinoma cell lines tested. However, in primary tumors with hypermethylation upstream of TSS, a decrease in $MST1R_{long}/MST1R_{total}$ ratio was not detected, although higher expression ratio of nuclear factor-κB was apparent. Furthermore, survival analysis demonstrated that $MST1R_{long}/MST1R_{total}$ ratio was independently associated with shorter disease-specific and disease-free survival, whereas $MST1R_{total}$ expression associated with shorter disease-specific survival. In conclusion, although promoter methylation patterns seem to determine *MST1R* global transcription regulation in renal cell carcinoma, other mechanisms might contribute to deregulate *MST1R* variant expression in RCT. Nevertheless, $MST1R_{total}$ expression and $MST1R_{long}/MST1R_{total}$ ratio modulate the biological and clinical aggressiveness of renal cell carcinoma, as depicted by its prognostic significance, a finding that requires validation in a larger independent series.

Keywords: Renal cell tumors, *MST1R*, *RON*, *MST1R* promoter methylation, MST1R expression, epigenetics

Introduction

The macrophage stimulating 1 receptor (*MST1R*), also known as RON (recepteur d'origine nantais), is a C-MET proto-oncogene family receptor tyrosine kinase [1]. Both *MST1R/RON* and its ligand, macrophage-stimulating protein (MSP) [2], are mapped at chromosome 3p21 [1, 3], and MSP binding triggers *MST1R* dimerization and subsequent activation [4]. This leads to downstream signaling activation of RAS-MAPK and PI-3K-AKT pathways [4], determining increased proliferation, survival and invasion [5], epithelial to mesenchymal transition (EMT) [6] and chemoresistance [7]. Since the nomenclature used for *MST1R/RON* varies in different references, we will follow the designation used in the original study whenever we consider that it prevents further confusion, but otherwise we will use *MST1R*.

MST1R is constitutively transcribed in epithelial cells, macrophages, osteoclasts and hematopoietic cells [8-12], and its signaling was reported to be altered in several human cancers, including those of the breast [13], lung [14], liver [15], ovary [16], colon [17], bladder [18] and nasopharynx [19].

In addition to ligand-induced dimerization, *MST1R* activation may be accomplished by

receptor overexpression, kinase domain activating mutations and generation of constitutively active *MST1R* variants [4, 20]. Most of these variants originate from full-length *MST1R* (*flRON*) alternative mRNA splicing (*RONΔ170*, *RONΔ165*, *RONΔ160*, *RONΔ155*), but may also be generated from protein truncation (*RONΔ110*, *RONΔ75*) and alternative transcription start site (short-form RON, *sfRON*, or *RONΔ55*) [21]. Some of these variants are constitutively active and thought to be oncogenic, including *RONΔ165*, *RONΔ160*, *RONΔ155* and *RONΔ110* [21].

Concerning alternative transcription start site, two *MST1R* transcripts are often found in both normal and neoplastic cells, named full-length *RON* (*flRON*) and short-form *RON* (*sfRON*) [22, 23]. Whereas *flRON* transcription is initiated through a classical promoter upstream transcription start site (TSS) and it is enhanced by hypoxia-inducible factor 1α (*HIF-1α*) [24], early growth response-1 (*Egr-1*) [25] and nuclear factor-κB (*NF-κB*) [25] in cancer cells, *sfRON* transcription is initiated at the codon that encodes for Met913, using an alternative intragenic promoter located between introns 8 and 10 [12, 22]. Scarce data is available on alternative transcription start site regulation, but it has been reported that methylation pattern of MST1R promoter associates with differential *flRON* and *sfRON* expression: hypermethylation at an area upstream of *MST1R* promoter, named 'island 1', was associated with absence of *flRON* and the presence of *sfRON* expression, whereas 'island 1' low or absent methylation was associated with concomitant *flRON* and *sfRON* expression [22]. It was also suggested that *sfRON* endogenous activity might be influenced by *flRON* expression, since a protein complex that is promptly degraded is formed when both *sfRON* and *flRON* are co-expressed [22]. Hence, when 'island 1' is hypermethyalted, *MST1R* homeostasis is shifted towards *flRON* null or low expression levels, and increased *sfRON* expression and activity. *sfRON* protein is constitutively active and its overexpression has been associated with an aggressive tumor phenotype: cancer cells grow faster, lose epithelial morphology, cease to form cell aggregates and become motile [23], features that promote local invasion and metastatic spread.

Despite *MST1R* signaling was found to be deregulated in several neoplasms [13-19, 22, 23], few studies have focused on *MST1R* promoter methylation [22], particularly in renal cell tumors (RCT). We have previously reported that *MST1R* promoter hypermethylation in renal cell tumors (RCT) was a sensitive and specific biomarker for clear cell renal cell carcinoma [26], and the 307 renal tumors available in the "Catalogue of somatic mutations in cancer" (COSMIC) dataset (cancer.sanger.ac.uk) were reported as highly methylated [27]. RCTs, a clinical, morphological, genetically and epigenetically heterogeneous group of tumors, comprise both benign [e.g., oncocytoma (RO)], and malignant [e.g., clear cell renal cell carcinoma (ccRCC), papillary RCC (pRCC) and chromophobe RCC (chRCC)] neoplasms, among which ccRCC is the most frequent (75%) and aggressive subtype, followed by pRCC (10%), and then chRCC (5%), the least aggressive subtype that rarely metastasizes [28, 29]. Although *MST1R* protein expression has been previously investigated in RCTs, it mainly focused on chRCC and RO [30, 31], and, thus, studies on *MST1R* mRNA expression deregulation through promoter methylation, as well as its biological and clinical impact are lacking. Thus, we aimed to characterize *MST1R* promoter methylation in RCT to investigate whether altered patterns might associate with different transcript variant expression in RCT primary tumors and cell lines, and how it might impact on tumor aggressiveness.

Material and methods

Patients and sample collection

Fresh-frozen tissue was prospectively collected, after informed consent, from 130 nephrectomy specimens at the Portuguese Oncology Institute - Porto (Portugal) between 2003 and 2007, comprising samples from ccRCC, pRCC, chRCC and oncocytoma (30 of each), and 10 morphologically normal kidney (cortical) tissue (from patients with upper urinary tract neoplasia not invading the renal parenchyma). Tissue samples were snap-frozen immediately after surgery, stored at -80°C and later cut in a cryostat. An H&E slide was performed before and after the sections used for nucleic acid extraction, to ensure at least 70% of neoplastic cells in the tumor samples and negligible inflammation in morphologically normal kidney samples.

Routine assessment of tumor classification (WHO), grading (Fuhrman) and staging (TNM)

Table 1. Primer sequences, amplicon size, and annealing temperatures for *MST1R* [GenBank: NM_002447] bisulfite sequencing (BSP), quantitative methylation specific PCR (QMSP) and expression

Primer set	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')	Amplicon size (bp)	Location [bp upstream (up) or downstream (down) TSS]	Annealing temp (°C)
BSP					
MST1R_B_1	GTTATTGAGGGTGTTGTTATTAAGTG	ACCTAACCCAAACCCTCC	264	612 up to 348 up	60
MST1R_B_2	AGGTGAAGGTATAGGAGTTAGG	AAATTCCTATAAAACCCAAATC	272	417 up to 145 up	60
MST1R_B_3	GGTAGGGATTTTTTAGGGTTT	CACCATAACCTATACCAAACCTC	210	33 up to 177 down	60
QMSP					
$MST1R_{up}$	TTAAGGGTCGGAAGAGTC	ATACACTAACGCTTAACGCTC	128	540 up to 412 up	60
$MST1R_{TSS}$	AGCGTTAGTGTATAGCGGC	TAAACAACGATCCCGACA	169	270 up to 101 up	60
Expression					
$MST1R_{total}$	GGCTGAGGTCAAGGATGTGCT	GCCTTTGCCAATGACTCGGT	73	-	62
$MST1R_{long}$	CTCTGGGGACCAGGTTTTC	ATGAAATGCCATGCCCTTAG[a]	93	-	62
NF-κB	GCTTAGGAGGGAGAGCCCT	CTGCCATTCTGAAGCCGGG	86	-	61

[a]Primer sequence from [23].

was performed for all tumor cases in formalin-fixed paraffin-embedded tissue [29, 32]. Relevant clinical data was collected from clinical charts.

This study was approved by the Institutional Review Board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute of Porto, Portugal (CES518/2010).

Cancer cell lines

Cell lines representative of ccRCC, two established from primary tumors (769-P, 786-0) and one from a metastatic site (Caki-1) were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were cultured according to the manufacturer's specifications, with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA) and antibiotics (100 units/mL penicillin G and 100 µg/mL streptomycin, Gibco), in a humidified atmosphere of 5% CO_2 at 37°C.

769-P and 786-0 cancer cell lines were subjected to treatment with the demethylating drug 5-aza-2'deoxycytidine (1 µM for 72 h). In parallel, the same cell lines were cultured without treatment for 72 h and harvested before confluence. Demethylating treatment was conducted in triplicate for both cell lines.

Nucleic acid extraction

Genomic DNA from fresh-frozen samples and cell lines was extracted as previously described [33]. In brief, DNA was digested overnight with proteinase K (20 mg/mL) in the presence of 10% SDS at 55°C, and then extracted with phenolchloroform and precipitated with 100% ethanol.

RNA extraction was performed as previously reported [34] both for fresh-frozen tissues and cell lines. Briefly, TRIzol® reagent (Invitrogen™, Carlsbad, CA, USA) was used to suspend the samples, chloroform (Merk Millipore, Darmstadt, Germany) was added to the lysed cells, and total RNA was then purified using Ambion® PureLink RNA Mini Kit (Invitrogen™, Carlsbad, CA, USA), according to manufacturer's recommendations. RNA purity ratios and concentration were measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was confirmed by electrophoresis.

Bisulfite modification and bisulfite sequencing

Bisulfite conversion of unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain as such, was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA), according to manufacturer's instructions. The modified DNA was eluted in 60 mL of water and stored at -80°C.

Subsequently, *MST1R* [GenBank: NM_002447] promoter was subjected to direct bisulfite sequencing in 5 samples: 1 ccRCC, 1 pRCC, 1 chRCC, 1 RO and 1 normal kidney. Primers were specifically designed to amplify fragments containing the *MST1R* promoter CpG "island 1"

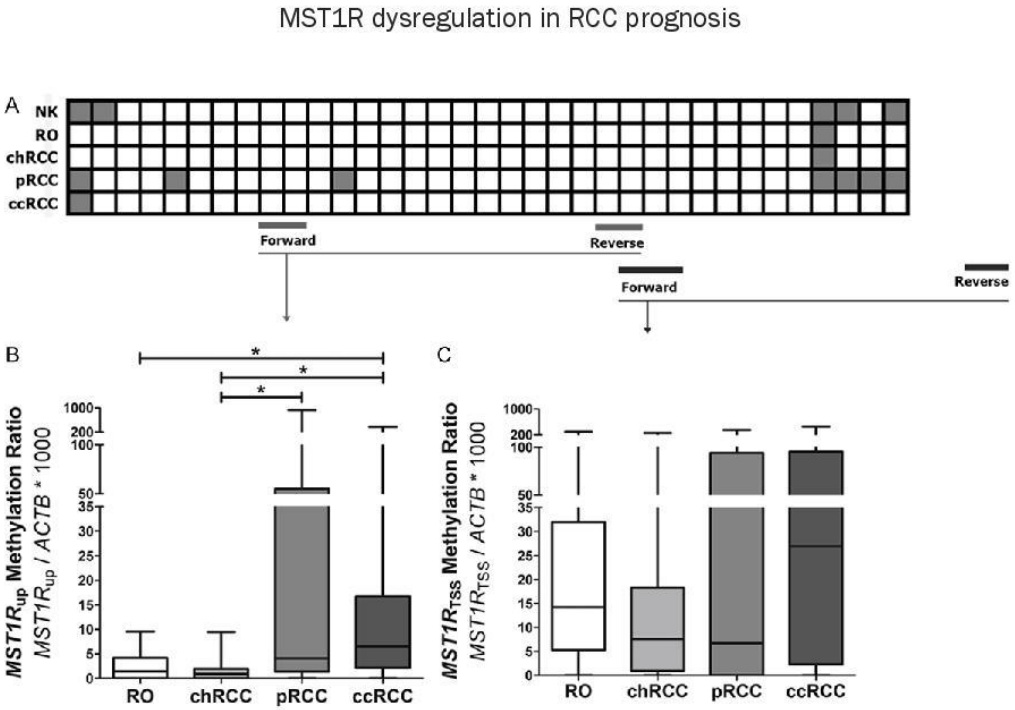

Figure 1. *MST1R* promoter methylation in renal cell tumors (RCT): bisulfite sequencing of *MST1R* promoter in 5 cases (A) and QMSP methylation levels in two distinct regions, one upstream TSS ($MST1R_{up}$) and another closer to TSS ($MST1R_{TSS}$), in 120 cases. White squares: CpG unmethylated; gray squares: CpG partially methylated. NK: normal kidney; RO: renal oncocytoma; chRCC: chromophobe renal cell carcinoma; pRCC: papillary renal cell carcinoma; ccRCC: clear cell renal cell carcinoma.

[22], using Methyl Primer Express v 1.0 (Applied Biosystems, Foster City, CA, USA). Primer sequences and location, amplicons, and annealing temperatures are listed in **Table 1**.

PCR reactions included a 94°C denaturation 10 min. step followed by 40 cycles at 94°C for 30 sec., annealing temperature for 30 sec., and 72°C for 30 sec. PCR products were loaded in a 2% agarose gel, stained with ethidium bromide and visualized under an ultraviolet transilluminator. Excess primer and nucleotides were removed by Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, USB Corporation, Cleveland, OH, USA) following manufacturer's protocol. The purified products were sequenced using the dGTP BigDye Terminator Cycle Sequencing ReadyReaction kit (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and data were analyzed by Sequencer Version 4.2.2 software. The peak height of the cytosine signal and the sum of the cytosine and thymine peak height signals were compared to calculate the approximate amount of methylcytosine of each CpG site. CpG sites with ratios 0-0.20, 0.21-0.80, and 0.81-1.0 were considered unmethylated, partially methylated, and fully methylated, respectively, as previously described [33, 35].

Quantitative MSP

Quantitative methylation specific real-time polymerase chain reaction (QMSP) was performed in cell lines before and after demethylating treatment, and in all frozen tissue samples, after DNA bisulfite conversion.

Primers were designed to specifically amplify methylated bisulfite converted complementary sequences of *MST1R* promoter using Methyl Primer Express v 1.0 (Applied Biosystems, Foster City, CA, USA), enclosing the region previously described as *MST1R* promoter "island 1" [22], located upstream of TSS [26]. Two areas were amplified, one upstream "island 1" but still in the *MST1R* promoter CpG island, and another downstream, more close to TSS, named $MST1R_{up}$ and $MST1R_{TSS}$ respectively (**Figure 1**). Primer sequences and location are listed in **Table 1**. β-*actin* (*ACTB*) was used as

reference gene to normalize for DNA input in each sample.

For QMSP analysis, a reaction volume of 20 µL consisting of 10 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 7 µL of H_2O, 0.5 µL of forward primer, 0.5 µL of reverse primer and 2 µL of bisulfate-modified DNA, was run in an 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Each sample was run in triplicate, a calibration curve was constructed with serial dilutions (1:5) of bisulfite converted universally methylated DNA at all CpGs (CpGenome Universal Methylated DNA; Millipore, Billerica, MA) to quantify the amount of fully methylated alleles in each reaction, and "no template controls" were included as a control for contamination. The amplification reaction was carried out at 95°C for 2 min followed by 45 cycles of 95°C for 15 s, and at annealing temperature (**Table 1**) for 1 min, followed by a melt curve.

Relative levels of methylated promoter DNA in each sample were determined by the ratio of the mean quantity obtained by QMSP analysis for each gene and the respective value of the internal reference gene (*ACTB*), multiplied by 1000 for easy tabulation (methylation level = target gene/reference gene × 1000).

Quantitative gene expression analysis

MST1R gene expression was evaluated in ccRCC cell lines before and after treatment when done, and in the 120 RCTs samples. For cell lines, 1 µg of total RNA was reversely transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems®, Foster City, CA, USA) according to manufacturer instructions. For frozen tissue, 300 ng of total RNA was reversely transcribed and amplified using TransPlex® Whole Transcriptome Amplification Kit (Sigma-Aldrich®, St. Louis, MO, United States) purified with QIAquick PCR Purification Kit (QIAGEN, Germany). Total *MST1R* expression ($MST1R_{total}$) and long form *MST1R* expression ($MST1R_{long}$) was evaluated using custom primers designed respectively to a region common to all *MST1R* described transcripts and to a region specific of the long form transcript (**Table 1**), using a Light Cycler 480 Real-time PRC system (Roche, Basel, Switzerland), in a reaction volume of 10 µL consisting of 5 µL of KAPA SYBR FAST® qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), 3.7 µL of H_2O, 0.15 µL of forward primer, 0.15 µL of reverse primer and 1 µL of cDNA.

Each sample was run in triplicate and the amount of cDNA was normalized to Glucuronidase beta (*GUSβ*) reference gene, as the ratio of the mean expression level obtained by QMSP analysis for each transcript and the respective value of the internal reference gene (*GUSβ*), multiplied by 1000 for easy tabulation. Each plate included multiple non-template controls and serial dilutions (1:5) of a cDNA Human Reference Total RNA (Agilent Technologies, La Jolla, CA, USA) to construct a standard curve.

NF-κB expression was evaluated in the 120 RCTs, as described above, using custom primers (**Table 1**).

Statistical analysis

Median and interquartile range of promoter methylation and expression levels were determined for cell lines and tumor samples. For tumor samples analysis and for each QMSP primer pair, each RCT sample was classified as "methylated" if the methylation level was higher than the highest value determined in the normal kidney samples ($MST1R_{up}$: 17.58; $MST1R_{TSS}$: 2.22), and as "not methylated" if the methylation level was lower than that value. $MST1R_{long/total}$ ratio was computed as the ratio $MST1R_{long}/MST1R_{total}$ x 100, after linear normalization of $MST1R_{total}$ relative expression [($MST1R_{total}$ value - $MST1R_{total}$ min)/($MST1R_{total}$ max - $MST1R_{total}$ min)] and $MST1R_{long}$ relative expression [($MST1R_{long}$ value - $MST1R_{long}$ min)/($MST1R_{long}$ max - $MST1R_{long}$ min)].

Non-parametric tests were used to ascertain the statistical significance of differences among groups of samples, namely Kruskal-Wallis ANOVA test (KW) for multiple comparisons and Mann-Whitney U test (MW) with Bonferroni's correction for pair-wise comparisons, as appropriate. Spearman's test was carried out to ascertain correlations between age and gene expression levels.

Prognostic significance of standard clinicopathological variables (histological subtype, pathological stage, Fuhrman grade, age, gender) and of $MST1R_{up}$ and $MST1R_{TSS}$ methylation level, $MST1R_{long/total}$ ratio, $MST1R_{total}$ and *NF-κB* expression levels, was assessed by constructing

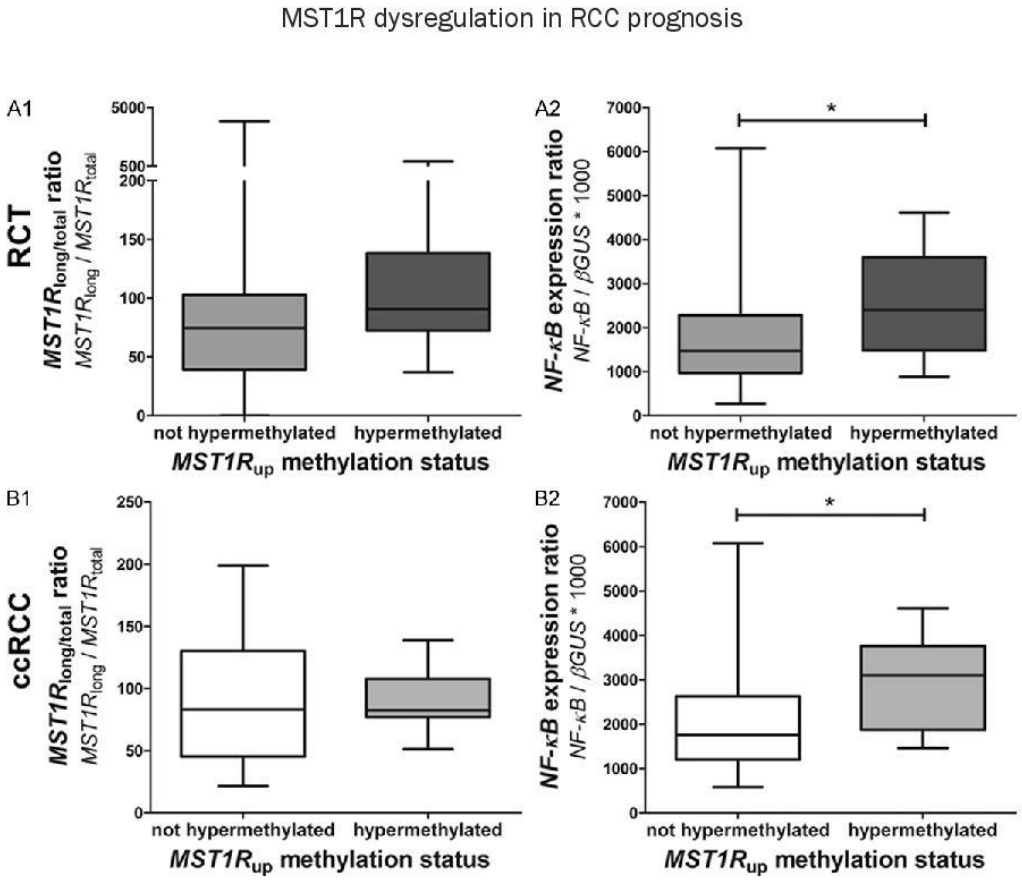

Figure 2. Expression levels in tumors hypermethylated or not at $MST1R_{up}$ (upstream area of *MST1R* promoter): $MST1R_{long/total}$ ratio in RCTs (n=120) (A1) and in ccRCC (n=30) (B1), and *NF-κB* expression ratio in RCTs (A2) and ccRCC (B2).

disease-specific and disease-free survival (defined, respectively, as the time between diagnosis and death for renal cell carcinoma, and the time between treatment and the first metastasis or local recurrence) curves using the Kaplan-Meier method, with log-rank test (univariable test). For this purpose, expression levels and ratio were classified as low or high using as cut-off the 75th percentile expression value of each gene/ratio. Multivariable survival analysis was conducted only for ccRCC and pRCC. The exclusion of chRCC from the analysis was due to the paucity of events (one patient presented progression/metastasis during follow-up and none has died from cancer). Age, stage and histological subtype were also included in the final Cox-regression model, both for disease-specific (DSS) and disease-free (DFS) survival.

Statistical significance was set at $p < 0.05$. Statistical analysis was performed using SPSS software for Windows, version 22.0 (IBM-SPSS Inc., Chicago, IL, USA), and graphs were built using GraphPad Prism 6.0 software for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

MST1R promoter methylation is higher near TSS in renal cell tumors

The methylation pattern of *MST1R* promoter in RCTs was characterized by QMSP using two primer sets, one upstream TSS ($MST1R_{up}$) and another more close to TSS ($MST1R_{TSS}$). Globally, $MST1R_{TSS}$ methylation levels [median (range): 14 (0-458)] was higher than those of $MST1R_{up}$ [median (range): 2 (0-933)], and 74% of samples were hypermethylated at $MST1R_{TSS}$ (22 ccRCC, 20 pRCC, 22 chRCC and 25 oncocytomas) whereas only 10% of samples were hypermethylated at $MST1R_{up}$ (4 ccRCC and 8 pRCC).

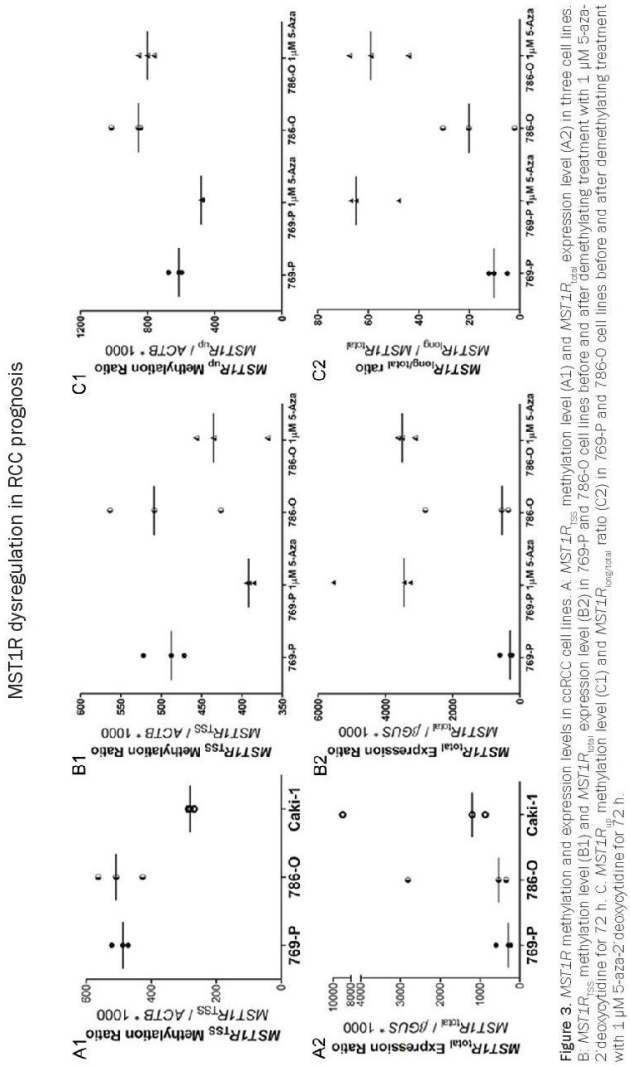

Figure 3. *MST1R* methylation and expression levels in ccRCC cell lines. A: $MST1R_{TSS}$ methylation level (A1) and $MST1R_{total}$ expression level (A2) in three cell lines. B: $MST1R_{TSS}$ methylation level (B1) and $MST1R_{total}$ expression level (B2) in 769-P and 786-O cell lines before and after demethylating treatment with 1 µM 5-aza-2'deoxycytidine for 72 h. C: $MST1R_{up}$ methylation level (C1) and $MST1R_{long/total}$ ratio (C2) in 769-P and 786-O cell lines before and after demethylating treatment with 1 µM 5-aza-2'deoxycytidine for 72 h.

MST1R dysregulation in RCC prognosis

Table 2. Clinical and pathological data of patients included in the present study

	Tumor	Normal
Number of patients, n	120	10
Age, median (range)	60 (29-83)	67.5 (20-83)
Gender, n (%)		
Male	73 (61)	7 (70.0)
Female	47 (39)	3 (30.0)
Histological subtype, n (%)		n.a.
Clear cell RCC	30 (25)	
Papillary RCC	30 (25)	
Chromophobe RCC	30 (25)	
Oncocytoma	30 (25)	
Pathological Stage, n (%)		n.a.
Stage I	47 (39)	
Stage II	19 (16)	
Stage III	21 (17.5)	
Stage IV	3 (2.5)	
n.a. (oncocytoma)	30 (25)	
Fuhrman grade, n (%)		n.a.
1	3 (2.5)	
2	28 (23)	
3	45 (37.5)	
4	14 (12)	
n.a.	30 (25)	

n.a.: not applicable.

This is in line with overall results of bisulfite sequencing in the 5 samples analyzed, which revealed rare methylated CpG in the $MST1R_{up}$ area, and an increase of methylated CpG dinucleotides near TSS (**Figure 1A**). Additionally, at $MST1R_{up}$, significantly higher methylation level were depicted for ccRCC and pRCC compared to chRCC, and for ccRCC compared to oncocytoma ($p<0.001$ for all) (**Figure 1B**). There were no statistically significant differences in $MST1R_{TSS}$ methylation levels among RCT subtypes ($p=0.291$) (**Figure 1C**).

NF-κB expression associates with $MST1R_{long/total}$ ratio in hypermethylated RCTs

RCTs with $MST1R_{TSS}$ hypermethylation showed a significantly lower $MST1R_{total}$ expression ratio ($p=0.049$), and RCTs with $MST1R_{up}$ hypermethylation displayed a trend for higher expression of $MST1R_{long/total}$, ($p=0.053$) (**Figure 2A**). Interestingly, a significantly higher expression of NF-κB ($p=0.013$) was also observed in these RCTs (**Figure 2B**).

When analyzing ccRCC and pRCC independently ($MST1R_{up}$ hypermethylation was not apparent in chRCC or oncocytomas), there were no differences in $MST1R_{long/total}$ in ccRCC with or without $MST1R_{up}$ hypermethylation ($p=0.756$) (**Figure 2C**), but ccRCC with $MST1R_{up}$ hypermethylation displayed a significantly higher NF-κB expression ($p=0.036$) (**Figure 2D**). No statistically significant differences were depicted for pRCC.

MST1R expression is regulated by promoter methylation pattern in ccRCC cell lines

MST1R promoter methylation levels more close to TSS ($MST1R_{TSS}$) and $MST1R_{total}$ expression was evaluated in 769-P, 786-O and Caki-1 ccRCC cell lines. $MST1R_{total}$ expression was lowest in 769-P and 786-O cells (**Figure 3A2**), which also displayed the highest $MST1R_{TSS}$ methylation levels (**Figure 3A1**), paralleling the observations in primary tumors. Demethylating treatment in those two cell lines restored $MST1R_{total}$ expression (**Figure 3B2**), mainly $MST1R_{long}$ (*fIRON*) expression, which was apparent through a higher $MST1R_{long/total}$ ratio (**Figure 3C2**), and decreased $MST1R_{TSS}$ and $MST1R_{up}$ methylation levels (**Figure 3B1** and **C1**).

Clinical-pathological associations and survival analysis

Clinical and pathological features of the 120 patients included in this study are depicted in **Table 2**. The methylation levels of $MST1R_{up}$ and $MST1R_{TSS}$, as well as $MST1R_{total}$ expression level, $MST1R_{long/total}$ ratio and *NF-κB* expression level, were not associated with gender ($p=0.563$, $p=0.263$, $p=0.561$, $p=0.159$ and $p=0.576$, respectively), age ($p=0.352$, $p=0.979$, $p=0.676$, $p=0.119$ and $p=0.056$, respectively) or pathological stage ($p=0.661$, $p=0.908$, $p=0.132$, $p=0.579$ and $p=0.822$, respectively).

A significantly lower *NF-κB* expression level ($p<0.001$) was observed in oncocytomas compared to RCC, whereas for $MST1R_{up}$ and $MST1R_{TSS}$ methylation levels, $MST1R_{total}$ expression levels and $MST1R_{long/total}$ ratio, no significant differences were found.

During follow-up [median (range): 60 months (2-392 months)], 12 (13%) patients died from RCC and 17 (19%) developed metastatic disease. Among molecular parameters, only $MST1R_{total}$ expression levels associated with

Figure 4. Kaplan-Meier analysis for disease-specific survival in 60 RCC patients, according to $MST1R_{total}$ expression level (A) and $MST1R_{long/total}$ ratio (B), and for disease-free survival in 60 RCC patients, according to $MST1R_{long/total}$ ratio (C). The results presented were categorized using third quartile (75th percentile) value as cutoff.

MST1R dysregulation in RCC prognosis

Table 3. Prognostic value of pathological stage, histological subtype and *MST1R* expression in renal cell carcinomas, following multivariable analysis using Cox-regression model

Prognostic Factor	Multivariable Analysis		
	Hazard Ratio (HR)	95% CI for HR	Cox regression *p* value
Disease Specific Survival[a]			
- Stage III/Stage IV (*vs* Stage I/Stage II)	38	5.4-269	< 0.001
- pRCC (*vs* ccRCC)	22	3.1-157	0.002
- High $MST1R_{total}$ expression level (*vs* low $MST1R_{total}$ expression level)	10	1-96	0.046
- Stage III/Stage IV (*vs* Stage I/Stage II)	26	4.4-153	< 0.001
- pRCC (*vs* ccRCC)	14.6	2.2-99	0.006
- Low $MST1R_{long}/MST1R_{total}$ ratio (*vs* high $MST1R_{long}/MST1R_{total}$ ratio)	4.9	1.2-20	0.025
Disease Free Survival[b]			
- Stage III/Stage IV (*vs* Stage I/Stage II)	14	3.5-59	< 0.001
- Low $MST1R_{long}/MST1R_{total}$ ratio (*vs* high $MST1R_{long}/MST1R_{total}$ ratio)	3.2	1.1-9.5	0.038

Only ccRCC and pRCC were included due to insufficient events in chRCC. CI: Confidence Interval; ccRCC: clear cell renal cell carcinoma; pRCC: papillary renal cell carcinoma. [a]Adjusted for patient age. [b]Adjusted for patient age and histological subtype; $MST1R_{total}$ expression level did not attained statistical significance in multivariable analysis for disease free survival.

development of metastasis during follow-up (p=0.049). Patients with a low RCC $MST1R_{total}$ expression displayed shorter DSS, and those with high $MST1R_{long/total}$ ratio presented shorter DSS and DFS (**Figure 4**), which was statistically significant in multivariable analysis, controlling for stage, histological subtype and age (**Table 3**).

Discussion

Gene expression regulation by promoter methylation is a well described epigenetic mechanism and its deregulation is considered an early event in carcinogenesis [36]. Indeed, aberrant promoter hypermethylation is associated with transcriptional repression [36, 37] and, thus, gene re-expression after demethylating treatment has been widely used as a strategy for identification of genes regulated by promoter methylation, namely in RCC [38-42]. *MST1R* promoter had been previously reported as hypermethylated in RCC in an area downstream of TSS [26, 27] and in the regions investigated by Angeloni and co-workers [22], but its putative association with altered *MST1R* expression pattern was not further explored. Our findings suggest that *MST1R* global expression ($MST1R_{total}$) is predominantly modulated by promoter methylation near TSS ($MST1R_{TSS}$), because significantly lower $MST1R_{total}$ expression was found in primary RCT with $MST1R_{TSS}$ hypermethylation, lower $MST1R_{total}$ expression was found in ccRCC cell lines with higher $MST1R_{TSS}$ methylation levels (769-P and 786-0), and $MST1R_{total}$ increased expression was observed in those cell lines after demethylating treatment.

It has been previously suggested that the pattern of promoter methylation was associated with the expression of different *MST1R* variants, specifically that the methylation of a particular promoter region upstream TSS - 'island 1' - was associated with lack of $flRON/MST1R_{long}$ and an increase of *sfRON* transcription, through an alternative internal promoter, with a consequent decrease in $MST1R_{long/total}$ ratio [22]. By bisulfite sequencing we found that not only the region previously described as 'island 1' but also its' upstream region within the CpG island were not methylated in RCTs, and thus we designed primers slightly upstream 'island 1' to further explore this *MST1R* promoter area. The quantification of *sfRON* expression could provide additional information concerning the variation of expression of different transcripts, but this was not possible due to the inability to design primers specific for *sfRON*. Surprisingly, a higher $MST1R_{long/total}$ ratio was found in RCTs with $MST1R_{up}$ hypermethylation (using a QMSP primer set specific to 'island 1'), although it did not reach statistical significance.

Because $MST1R_{long}$ is under control of the classical *MST1R* promoter, we hypothesized that transcription factors acting on *MST1R* in cancer cells might contribute to $MST1R_{long}$ expres-

sion, overcoming the methylation inhibitory effect. Since *NF-κB* has more predicted binding sites in the MST1R promoter than *HIF-1α* and *Egr-1*, *NF-κB* expression was determined in RCTs and, indeed, we found that RCTs with $MST1R_{up}$ hypermethylation displayed a significantly higher level of *NF-κB* expression, suggesting that promoter methylation is not the sole mechanism regulating *MST1R* expression.

Nevertheless, aberrant promoter methylation seems to be a relevant cause of *MST1R* silencing, because in 769-P and 786-O cells the $MST1R_{long/total}$ ratio increased after demethylating treatment. Importantly, increase in *fIRON* expression after demethylating treatment had already been reported for other cell lines, including TF1 (erythroleukemia) and lung cancer cell lines [22].

We have previously reported that promoter methylation in a region downstream $MST1R_{TSS}$ identifies ccRCC with high sensitivity and specificity [26]. Similar diagnostic performance was not demonstrated for methylation of $MST1R_{up}$ or $MST1R_{TSS}$, neither for $MST1R_{total}$ expression or $MST1R_{long/total}$ ratio. Nevertheless, the present study demonstrated that lower $MST1R_{total}$ expression and higher $MST1R_{long/total}$ ratio independently predict worse prognosis in ccRCC and pRCC. Intriguingly, in urothelial carcinoma of the bladder, MST1R protein expression was associated with a worse prognosis [18] and *MST1R* overexpression is one of the mechanisms for activation of *MST1R* signaling, which seems to confer a more aggressive phenotype to cancer cells. However, it should be taken in account that in our series, *MST1R* overexpression is not a common alteration driving activation of signaling pathways that lead to cancer cell proliferation, invasion and metastization in RCC. On the contrary, we found significantly lower $MST1R_{total}$ expression in association with $MST1R_{TSS}$ hypermethylation in RCTs. Indeed, this contrasts with the more prominent role of *MST1R* in other cancer models, including nasopharyngeal carcinoma (NPC), in which latent membrane protein 1 (LMP1) stimulates NF-κB binding to *MST1R* promoter, inducing EMT, a finding that may explain the higher metastatic potential of NPC with LMP1 overexpression [19].

It should, however, be noted that the biological interpretation of *MST1R* expression in RCT primary tumor is not straightforward. We explored the association of *MST1R* promoter methylation pattern and $MST1R_{long/total}$ ratio, and given that *sfRON* is a constitutively active variant with oncogenic potential, it would be expectable that most aggressive tumors should display a lower $MST1R_{long/total}$ ratio. However, some $MST1R_{long}$ splicing variants have oncogenic potential, and even the overexpression of *MST1R* could lead to the activation of cell signaling pathways related to proliferation and metastization. The presence of such splicing variants, although functionally relevant for the understanding of *MST1R* role in renal carcinogenesis, was not further explored mainly because all are transcribed from the classical promoter and the $MST1R_{long}$ primer set was unable to discriminate splicing variants. Other *MST1R* activating mechanisms might also be relevant but their relative contribution might be limited. Indeed, the frequency of activating point mutations for RCC reported in COSMIC dataset (cancer.sanger.ac.uk) is low (3/1474, 0.2%), and the same holds true for the frequency of copy number variations (loss in 8/417, 1.9%) [27].

In conclusion, although promoter methylation patterns seem to determine *MST1R* global transcription regulation in renal cell carcinoma, other mechanisms might contribute to deregulate *MST1R* variant expression in RCT. Nevertheless, $MST1R_{total}$ expression and $MST1R_{long}$/$MST1R_{total}$ ratio modulate the biological and clinical aggressiveness of renal cell carcinoma, as depicted by its prognostic significance, a finding that requires validation in a larger independent series.

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Disclosure of conflict of interest

None.

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CHAPTER 5

HISTONE MODIFYING ENZYMES IN RENAL CELL TUMORS

5.1. CHAPTER OVERVIEW

The results presented in this chapter are:

- *published in an international peer reviewed journal* [***Pires-Luís AS****, Vieira-Coimbra M*, Vieira FQ, Costa-Pinheiro P, Silva-Santos R, Dias PC, Antunes L, Lobo F, Oliveira J, Gonçalves CS, Costa BM, Henrique R, Jerónimo C. Expression of histone methyltransferases as novel biomarkers for renal cell tumor diagnosis and prognostication. Epigenetics, 2015; 10 (11):1033-43 (*joint first authors)*];
- *accepted for publication in an international peer reviewed journal* [*Ferreira MJ*, **Pires-Luís AS***, Vieira-Coimbra M, Costa-Pinheiro P, Antunes L, Dias PC, Lobo F, Oliveira J, Gonçalves CS, Costa BM, Henrique R, Jerónimo C. SETDB2 and RIOX2 are differentially expressed among renal cell tumor subtypes, associating with prognosis and metastization. Epigenetics, in press (*joint first authors)*].

RATIONALE

Histone modifying enzymes and other epigenetic modulators, as SWI/SIFT components, were found to be altered in renal cell carcinoma, mostly by mutation [1, 2], highlighting chromatin remodeling as a key component in renal tumorigenesis [3]. Furthermore, some histone methylation marks were associated with prognosis in renal cell tumors [4, 5], but the expression pattern of the enzymes that methylate or demethylate histones remains largely unexplored, with few exceptions, as for UTX and JMJD3 [6].

MAJOR FINDINGS

- Identification and validation of five differentially expressed histone demethylases and methyltransferases in renal cell tumors – SMYD2, SETD3, NO66, SETDB2 and RIOX2.
- Additional validation in TCGA dataset globally similar to our series
- Low expression levels of histone modifying enzymes were associated with worse prognosis in renal cell carcinoma:
    - Low SMYD2, SETD3 and NO66 were associated with shorter disease-specific survival (multivariable analysis)
    - Low SMYD2, SETD3, NO66 and SETDB2 were associated with shorter disease-free survival (multivariable analysis)
- RIOX2 expression level was associated with the development of metastasis during follow-up.

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5.2. EXPRESSION OF HISTONE METHYLTRANSFERASES AS NOVEL BIOMARKERS FOR RENAL CELL TUMOR DIAGNOSIS AND PROGNOSTICATION

RESEARCH PAPER

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Expression of histone methyltransferases as novel biomarkers for renal cell tumor diagnosis and prognostication

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Keywords: epigenetic biomarkers, histone covalent modifications, histone methyltransferases, NO66, Renal cell tumors, SMYD2, SETD3

Renal cell tumors (RCTs) are the most lethal of the common urological cancers. The widespread use of imaging entailed an increased detection of small renal masses, emphasizing the need for accurate distinction between benign and malignant RCTs, which is critical for adequate therapeutic management. Histone methylation has been implicated in renal tumorigenesis, but its potential clinical value as RCT biomarker remains mostly unexplored. Hence, the main goal of this study was to identify differentially expressed histone methyltransferases (HMTs) and histone demethylases (HDMs) that might prove useful for RCT diagnosis and prognostication, emphasizing the discrimination between oncocytoma (a benign tumor) and renal cell carcinoma (RCC), especially the chromophobe subtype (chRCC). We found that the expression levels of 3 genes—*SMYD2, SETD3*, and *NO66*—was significantly altered in a set of RCTs, which was further validated in a large independent cohort. Higher expression levels were found in RCTs compared to normal renal tissues (RNTs) and in chRCCs comparatively to oncocytomas. *SMYD2* and *SETD3* mRNA levels correlated with protein expression assessed by immunohistochemistry. *SMYD2* transcript levels discriminated RCTs from RNT, with 82.1% sensitivity and 100% specificity [area under curve (AUC) = 0.959], and distinguished chRCCs from oncocytomas, with 71.0% sensitivity and 73.3% specificity (AUC = 0.784). Low expression levels of *SMYD2, SETD3*, and *NO66* were significantly associated with shorter disease-specific and disease-free survival, especially in patients with non-organ confined tumors. We conclude that expression of selected HMTs and HDMs might constitute novel biomarkers to assist in RCT diagnosis and assessment of tumor aggressiveness.

Introduction

Kidney cancer is the most lethal of the common urological cancers, with 337,860 new cases and 143,369 deaths registered worldwide in 2012.[1] In the United States, 63,920 new cases and 13,860 deaths due to kidney cancer were estimated in 2014.[2] Renal cell tumors (RCTs), which originate from renal tubule cells, are the most frequent kidney neoplasms, accounting for 85% to 90% of all cases.[3] RCTs are a heterogeneous group of neoplasms, comprising several different histological subtypes, each with distinct morphologic, genetic, and clinical features. The four major RCT subtypes include 3 malignant tumors [clear cell renal cell carcinoma (ccRCC, the most common subtype, 75% of all RCT), papillary RCC (pRCC, 10% of all RCT), and chromophobe RCC (chRCC, 5% of all RCT)] and a benign tumor (renal oncocytoma), comprising 3 to 5% of all adult RCT.[3] Due to their different clinical aggressiveness, accurate classification is required for appropriate patient management.

The widespread use of imaging techniques has increased the detection of small renal masses, requiring novel tools for accurate diagnosis. Currently, RCT diagnosis relies on histopathological examination of biopsy or surgical specimens, a task that is challenging owing to the overlapping features of some tumor subtypes. One of the most common diagnostic problems is the

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differential diagnosis between renal oncocytoma and chRCC, especially its eosinophilic variant.[4,5] Although these tumors share some morphologic and imagiological features, they are biologically distinct, as chRCCs constitute low-grade malignant neoplasms that sometimes behave aggressively and carry a risk of recurrence and metastization, whereas oncocytomas are benign tumors, that might be more conservatively managed.[6] Among RCC, prognostication is also a challenging task. Although pathological stage, nuclear grade, histologic subtype and performance status are commonly used in clinical practice,[7] their ability to accurately predict tumor behavior is limited.[8,9]

Epigenetic alterations, comprising aberrations in DNA methylation patterns, deregulated chromatin machinery, and non-coding RNAs expression, play a critical role in neoplastic transformation, including renal carcinogenesis.[10-12] The modulation of chromatin conformation through covalent posttranslational histone modifications is a fundamental mechanism of gene transcription regulation, which includes methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation of specific residues.[13] Specifically, histone methylation, occurring at the side chains of lysine or arginine residues, is a dynamic process mediated by histone methyltransferases (HMTs) and histone demethylases (HDMs). More than 50 HMTs and HDMs have been identified thus far and, unlike other histone modifications, methylation does not change the charged state of the residues and, therefore, the effect on gene expression is dependent on the residue and its methylation level (mono-, di-, or tri-methylation).[14,15] In RCTs, the deregulation of chromatin machinery has been increasingly acknowledged as an important mechanism of neoplastic transformation (comprehensively reviewed in[16]), highlighting its potential role as diagnostic and prognostic biomarker.

The main goal of this study was to identify HMTs and HDMs that might be used as biomarkers to assist in diagnosis and prognosis of RCT. For that purpose, we screened 87 HMTs and HDMs genes for differential expression between normal renal tissue and RCT, as well as among RCT subtypes, with a particular emphasis on the discrimination of chRCC from oncocytoma. Differentially expressed genes were then validated in a large series of RCTs and renal normal tissues (RNT). Finally, its clinical usefulness as biomarkers was assessed and compared with standard clinicopathological parameters.

Results

Screening of histone methyltransferases and demethylases

Globally, the analysis of 58 HMTs and 29 HDMs expression levels in 5 chRCCs, 5 oncocytomas and 5 RNTs, disclosed HMTs upregulation in RCTs compared to RNTs. Conversely, HMTs and HDMs were generally downregulated in chRCCs compared to oncocytomas (**Fig. S1**). However, only *SMYD2* ($P = 0.01$), *SETD3* ($P = 0.005$), and *NO66* ($P = 0.014$) showed significantly higher expression levels in RCTs compared to RNTs, and were simultaneously overexpressed in chRCCs in comparison to oncocytomas.

Validation of selected genes

Validation of *SMYD2, SETD3* and *NO66* by RT-qPCR in a series of 160 RCTs and 10 RNTs confirmed that these 3 enzymes were significantly overexpressed in RCTs compared to RNTs ($P < 0.001$ for *SMYD2* and *SETD3, P* = 0.001 for *NO66*; **Fig. 1A**, 1-3). Additionally, expression levels of *SETD3* and

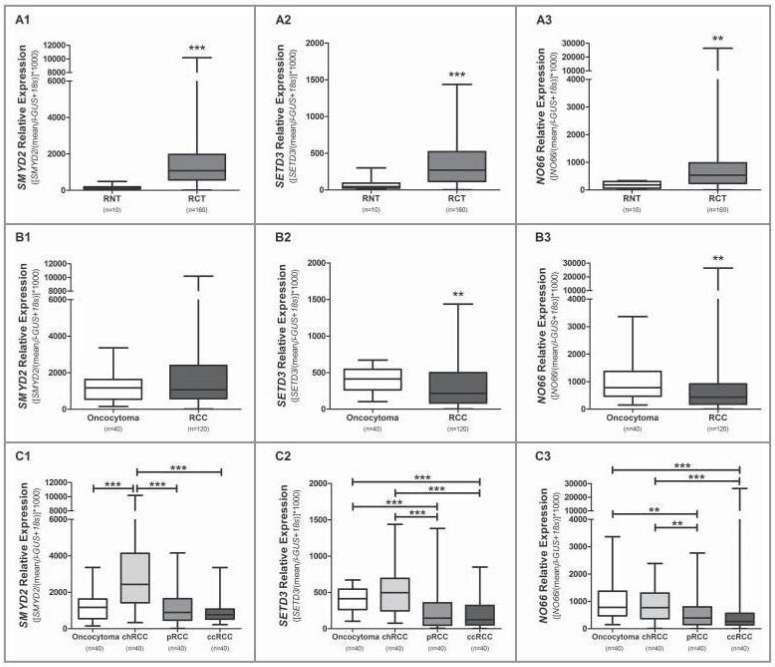

Figure 1. Distribution of expression levels of selected genes. Comparison between RCTs and RNTs for *SMYD2* (A1), *SETD3* (A2), and *NO66* (A3). Benign tumors *versus* malignant tumors for *SMYD2* (B1), *SETD3* (B2), and *NO66* (B3). Distribution of *SMYD2* (C1), *SETD3* (C2), and *NO66* (C3) expression levels according to renal cell tumor subtype. (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$).

NO66 differed significantly between benign and malignant RCTs ($P = 0.003$ and $P = 0.001$, respectively; **Fig. 1B**, 1-3). Among the 4 RCT subtypes analyzed, chRCC displayed the highest expression levels for the 3 genes, followed by oncocytoma and then by pRCC and ccRCC (**Fig. 1C**, 1-3). Pairwise comparisons showed statistically significant differences of *SMYD2, SETD3*, and *NO66* expression, in chRCC vs. pRCC ($P < 0.001$, $P < 0.001$, and $P = 0.004$, respectively) and chRCC vs. ccRCC ($P < 0.001$ for all), as well as for *SETD3* and *NO66* expression in oncocytoma vs. pRCC ($P < 0.001$ and $P = 0.001$, respectively) and oncocytoma vs. ccRCC ($P < 0.001$ for both). Additionally, *SMYD2* expression levels differed significantly ($P < 0.001$) between chRCC vs. oncocytoma (**Fig. 1C**, 1-3), in this validation series.

Correlation analysis for SMYD2, SETD3, and NO66 expression in RCC revealed that these 3 genes were significantly co-expressed (SMYD2 – SETD3: r = 0.759; SMYD2 – NO66: r = 0.639; SETD3 – NO66: r = 0.741; $P < 0.001$ for all).

Association between gene expression and clinicopathological features

Clinical and pathological features of patients included in this study are depicted in **Table 1**. No significant differences in gender were apparent between RCT patients and controls ($P = 0.524$). In RCTs, *NO66* expression levels were significantly higher in females ($P = 0.044$) and *SMYD2* expression levels were associated with patient's age ($P = 0.031$). In malignant tumors, no statistically significant associations were disclosed between *SMYD2, SETD3*, or *NO66* expression levels and pT (**Table S1**, **Fig. S2**), nor with pathological stage ($P = 0.692$, $P = 0.724$ and $P = 0.843$, respectivelly). *SMYD2* and *SETD3* expression levels were significantly higher in Fuhrman grade 1/2 vs. 3/4 ccRCCs and pRCCs ($P = 0.045$ and $P = 0.021$, respectively; **Table S2**, **Fig. S3**). Fuhrman grading was not applied to chRCCs because this grading system does not reflect chRCC clinical aggressiveness and an alternative grading system has been proposed.[17]

Diagnostic performance of selected genes

The diagnostic performance of *SMYD2, SETD3*, and *NO66* mRNA expression was assessed in 3 different settings, using ROC curve analysis: (i) identification of RCTs vs. renal normal tissue; (ii) discrimination of malignant from benign RCTs; and (iii) distinction of chRCC from oncocytoma (**Table 2**). Whereas *SMYD2* expression levels discriminated RCTs from normal kidney (80.6% sensitivity, 100% specificity, AUC = 0.961; **Table 2** and **Fig. 2**), the performance of all 3 genes in distinction of malignant from benign tumors was modest (highest AUC = 0.671, for *NO66*) (**Table 2**). Finally, *SMYD2* expression levels could distinguish chRCCs from oncocytomas (AUC = 0.794) with 72.5% sensitivity and 72.5% specificity (**Table 2**).

Survival analysis

The median follow-up of RCC patients was 167 months (range: 1-391 months). When considering the 7 years period defined for survival analysis, 10 patients died and 13 developed metastasis. Patients with kidney-confined tumors (Stage I and Stage II) displayed a

Table 1. Clinical and pathological data of patients included in the present study

	Tumor	Normal
Number of patients, n	160	10
Age, median (range)	61 (29-86)	67.5 (20-83)
Gender, n (%)		
Male	92 (57.5)	7 (70.0)
Female	68 (42.5)	3 (30.0)
Histological subtype, n (%)		n.a.
Clear cell RCC	40 (25.0)	
Papillary RCC	40 (25.0)	
Chromophobe RCC	40 (25.0)	
Oncocytoma	40 (25.0)	
pT, n (%)		n.a.
pT1	68 (42.5)	
pT2	23 (14.4)	
pT3	29 (18.1)	
pT4	0 (0.0)	
n.a. (oncocytoma)	40 (25)	
Pathological stage, n (%)		n.a.
I	68 (42.5)	
II	23 (14.4)	
III	25(15.6)	
IV	4 (2.5)	
n.a. (oncocytoma)	40 (25)	
Fuhrman grade, n (%)		n.a.
1	3 (1.9)	
2	41 (25.6)	
3	58 (36.3)	
4	18 (11.3)	
n.a.	40 (25.0)	
Metastasis, n (%)	13 (10.8)	n.a.
Clear cell RCC	4 (30.8)	
Papillary RCC	7 (53.8)	
Chromophobe RCC	2 (15.4)	

RCC, Renal Cell Carcinoma; n.a., not applicable

Table 2. Validity estimates for each enzyme as a tissue biomarker

	(%)	SMYD2 (%)	SETD3 (%)	NO66 (%)
	SE	80.6	85	65.63
	SP	100	80	100
	PPV	100	98.6	100
RCT vs. normal renal tissue	NPV	24.4	25.0	15.4
	Accuracy	81.8	84.7	67.6
	AUC	96.1	83.6	81.7
	SE	40.8	60.0	60.0
	SP	65.0	65.0	67.5
	PPV	77.8	83.7	84.7
RCC vs. oncocytoma	NPV	26.8	35.1	36.0
	Accuracy	46.9	61.3	61.9
	AUC	54.2	66.0	67.1
	SE	72.5	45.0	50.0
	SP	72.5	85.0	57.5
	PPV	72.5	75.0	54.1
chRCC vs.oncocytoma	NPV	72.5	60.7	53.5
	Accuracy	72.5	65.0	53.8
	AUC	79.4	59.1	52.3

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; AUC, area under the curve; RCT, renal cell tumor; RCC, renal cell carcinoma; chRCC: chromophobe RCC

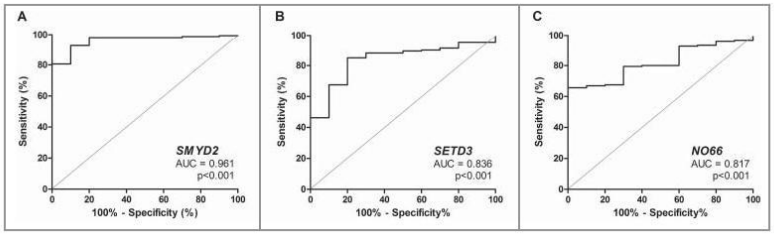

Figure 2. Receiver operator characteristic (ROC) curves evaluating performance of *SMYD2* (**A**), *SETD3* (**B**), and *NO66* (**C**) expression levels as biomarkers for discrimination between RCTs and RNTs (AUC: area under the curve).

significantly higher disease-specific survival (DSS) ($P < 0.001$, respectively); however, no statistically significant difference was apparent for DFS ($P = 0.055$). DSS ($P = 0.018$), but not DFS, was also significantly different among the 3 RCC subtypes. DSS and DFS did not associate with age, gender, or Fuhrman grade for the 3 RCC subtypes in this series. However, considering only ccRCC and pRCC, Fuhrman grade 4 was associated with lower DSS ($P < 0.001$) and DFS ($P < 0.001$).

Focusing on the expression levels of the validated enzymes, survival analysis showed that low *SMYD2, SETD3*, and *NO66* expression levels were significantly associated with shorter DSS ($P = 0.012$, $P = 0.001$, and $P = 0.011$, respectively; **Fig. 3A**) and DFS ($P < 0.001$, $P < 0.001$ and $P = 0.001$, respectively, **Fig. 3B**). When evaluating each enzyme's expression level and pT stage in multivariate analysis (pT and *SMYD2* expression level; pT and *SETD3* expression level; pT and *NO66* expression level), low levels of all 3 genes and pT3 were significantly associated with shorter DSS, and low SMYD2 expression levels and pT3 were significantly associated with shorter DFS (**Table S3**). Similar results were depicted for pathological stage (pTNM; data not shown). Conversely, in a model comprising gender, histological subtype, pathological stage, *SMYD2, SETD3*, and *NO66* expression levels simultaneously, only patients with tumors not confined to the kidney (Stage III / Stage IV) and that presented metastasis during the follow-up were found to have a significantly increased risk of death due to RCC. Moreover, patients with lower *SMYD2* expression levels and tumors not confined to the kidney (Stage III / Stage IV) presented a significantly increased risk of RCC progression (**Table 3**).

Immunohistochemical evaluation of SMYD2 and SETD3 expression

Immunoreactivity for SMYD2 and SETD3 was observed in the cytoplasm. In normal renal parenchyma, weak to moderate SMYD2 and SETD3 expression was found in tubular epithelial cells. Oncocytomas and chRCC globally displayed higher staining intensity and/or percentage of positive cells, compared to ccRCC and pRCC, following the trend depicted for mRNA expression levels. Indeed, a significant association was documented between mRNA relative expression (RT-qPCR) and protein expression (immunohistochemistry) classified as high and low, both for SMYD2 ($P = 0.002$) and SETD3 ($P = 0.008$) (**Fig. S4**). However, SMYD2 and SETD3 immunoreactivity was not associated with Fuhrman grade ($P = 0.403$ and $P = 0.110$, respectively) in ccRCC and pRCC or with pathological stage ($P = 0.636$ and $P = 0.609$, respectively), DSS ($P = 0.599$ and $P = 0.3$ respectively), and DFS ($P = 0.99$ and $P = 0.192$, respectively) in RCC.

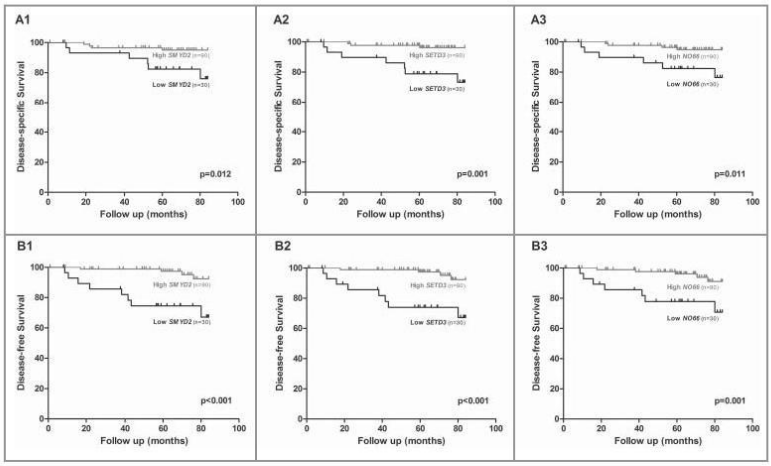

Figure 3. Kaplan-Meier with log-rank test estimates of disease-specific survival in 62 RCC patients according to expression levels of *SMYD2* (A1), *SETD3* (A2), and *NO66* (A3); and of disease-free survival in 88 RCC patients according to expression levels of *SMYD2* (B1), *SETD3* (B2) and *NO66* (B3). The results of RT-qPCR presented were categorized using first quartile (25th percentile) value as cutoff.

RNA expression and survival analysis of renal cell carcinoma patients from TCGA

Further validation of the results depicted for *SMYD2, SETD3* and *NO66* expression was performed in a larger and independent dataset from TCGA, including RNA-seq expression data from 889 RCC patients (533 ccRCC, 290

Table 4. Prognostic factors for TCGA dataset (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm. Accessed 2014) by Cox regression analysis

Prognostic Factor	Univariate Analysis			Multivariate Analysis		
	HR	95% CI for HR	*P* value[∞]	HR	95% CI for HR	*P* value[∞]
Disease Specific Survival						
Pathological Stage[§]	4.81	3.61-6.42	<0.0001	3.00	2.14-4.22	<0.0001
Metastasis (during follow-up)	6.14	4.59-8.20	<0.0001	2.62	1.86-3.70	<0.0001
SMYD2 expression level[*]			0.143			0.159
SETD3 expression level[*]	2.58	1.95-3.40	<0.0001	1.85	1.39-2.46	<0.0001
NO66 expression level[*]			0.220			0.850
Histological Subtype[δ]						
chRCC	—	—	<0.0001	—	—	0.047
pRCC	1.97	0.97-4.01	0.063	2.54	1.06-6.12	0.037
ccRCC	3.48	1.83-6.61	<0.0001	2.83	1.24-6.48	0.014
Gender[ψ]			0.625			0.196
Disease Free Survival						
Pathological Stage[§]	5.79	2.23-15.00	<0.0001	7.51	2.77-20.37	<0.0001
SMYD2 expression level[*]			0.809			0.751
SETD3 expression level[*]	3.91	1.48-10.36	0.006			0.100
NO66 expression level[*]	4.42	1.62-12.06	0.004	4.74	1.30-17.26	0.018
Histological Subtype[δ]						
chRCC	—	—	0.882			0.707
pRCC	$1.4e^{5}$	$0\text{-}7.5e^{108}$	0.923			0.921
ccRCC	$1.8e^{5}$	$0\text{-}9.7e^{108}$	0.921			0.925
Gender[ψ]			0.972			0.449

HR: Hazard Ratio; CI: Confidence Interval, n.s.: not significant.
[∞]Cox regression p value; significant when $P < 0.05$.
[§]Stage I / Stage II *vs* Stage III / Stage IV; reference group: Stage I / Stage II.
[*]Reference group: high expression level.
[δ]chRCC: chromophobe Renal Cell Carcinoma vs. pRCC: papillary Renal Cell Carcinoma vs. ccRCC: clear cell Renal Cell Carcinoma; Reference group: chRCC.
[Φ]Reference group: Absence of metastasis.
[ψ]Reference group: Male

The median follow-up of RCC patients from TCGA was 29.4 months (range: 0-195 months), in which 212 patients died and 92 developed metastasis. A significantly higher risk of death due to RCC was detected in ccRCC patients with non-organ confined tumors (Stage III and Stage IV), lower SETD3 expression, that developed metastasis during follow-up and with ccRCC and pRCC (compared to chRCC), in multivariate analysis ($P < 0.0001$; **Table 4**). Interestingly, Stage III / Stage IV tumors and lower NO66 expression levels associated with shorter time to disease progression in multivariate analysis ($P < 0.001$ and $P = 0.018$, respectively) (**Table 4**).

When analyzing each subtype separately, multivariate analysis revealed that Stage III / Stage IV was associated with shorter DSS in all subtypes and shorter DFS in ccRCC and pRCC; occurrence of metastasis during follow-up with shorter DSS in ccRCC and pRCC; low SETD3 expression levels with shorter DSS in ccRCC and pRCC and with shorter DFS in pRCC; and low NO66 expression levels with shorter DSS in pRCC (**Table S5**). DFS analysis was not performed to chRCC due to insufficient available data in the TGCA database.

Discussion

The incidental diagnosis of small renal masses has increased in recent years, and current imaging techniques, even when complemented with biopsy, are limited in discriminating benign from malignant entities, leading to surgical intervention in cases in which it might be spared and the patient be managed conservatively. Epigenetic-based biomarkers, including histone posttranslational modifications and chromatin modulators, hold the promise to assist in diagnosis through the discrimination between normal and neoplastic tissue or between benign and malignant tumors, helping in prognostication and stratifying patients according to risk of disease progression. Evidence of widespread deregulation of chromatin status in RCTs has been accumulating, and several defects in epigenetic enzymes, including those responsible for chromatin packaging, histone modifications, and chromatin remodeling, have been reported.[18,19]

In this study, we focused on altered histone methylation patterns and screened the expression of HMTs and HDMs genes in RNT, oncocytomas and chRCCs to determine whether their expression levels might be used as diagnostic biomarkers to discriminate normal from neoplastic renal tissue, as well as benign (oncocytoma) from malignant RCTs (especially chRCC). For most of the genes assessed, differences in expression levels between normal and neoplastic samples were in line with previous reports on their role in cancer. Thus, *SETD2*, a putative tumor suppressor in ccRCC,[20] was mostly downregulated in RCT compared to RNTS, and a similar trend was apparent for *KDM5C*, also reported to have a tumor suppressive effect.[21] *KDM6A*, previously reported to be overexpressed in RCTs,[22] was found to be the second most upregulated

gene in RCTs in our analysis. A divergent behavior was, however, observed for *EZH2*, previously shown to be upregulated in ccRCCs compared to adjacent normal tissues,[22,23] whereas we found *EZH2* downregulation in RCTs. Notwithstanding this latter result, which might be due to the small number of cases or the main histological subtype (chRCC) used for array analysis in our study, and/or the use of adjacent morphologically normal renal tissue as control (which we have found to harbor epigenetic alterations[24]) in the aforementioned studies, the overall results of the array and the agreement found for the 3 genes validated in a series of 160 primary tumors, as well as in GEO database (http://www.ncbi.nlm.nih.gov/gds) published array data,[25] argue in favor of the validity of our strategy for discovery of HDMs and HMTs as RCT biomarkers.

We found that 2 histone methyltransferases, *SMYD2* and *SETD3*, and a histone demethylase, *NO66*,[14] were significantly upregulated in RCTs compared to RNTs, and in chRCC compared to oncocytoma, in the screening array. SMYD2 targets lysines 4 and 36 of histone H3, as well as non-histone proteins including p53, RB1, HSP90, and PARP-1.[26-34] It plays an important role in muscle function, mediating the methylation of Hsp90, which stabilizes the sarcomeric region,[29] and in the early stages of embryonic differentiation.[35] In cancer, *SMYD2* is overexpressed in bladder cancer[36] and leukemia,[37] as well as in esophageal squamous cell carcinoma and gastric carcinoma.[38,39] *SETD3*, which also methylates lysines 4 and 36 of histone H3, promotes differentiation of muscle cells[40] and is overexpressed in lymphomas, displaying oncogenic potential.[41] *NO66*, which specifically targets lysines 4 and 36 of histone H3,[42] is involved in bone differentiation (osteoblast differentiation and bone formation),[43] inducing chromatin repression through histone demethylation during osteoblast differentiation.[44]

Our screening data are in line with "GEO Data sets" publically available expression array data (http://www.ncbi.nlm.nih.gov/gds) of 18 cases, comprising 9 chRCC and 9 oncocytomas,[25] which also revealed a higher expression of *SMYD2, SETD3*, and *NO66* in chRCC compared to oncocytoma (**Table S6**). Importantly, this pattern of expression was also retained in our validation series: chRCC and oncocytoma displayed higher expression levels than ccRCC and pRCC. This finding might denote the common cellular origin of ccRCC and pRCC, on the one hand, and of chRCC and oncocytoma, on the other, as previously suggested.[45] Indeed, expression levels of these 3 enzymes were significantly different between chRCC and pRCC, as well as between chRCC and ccRCC, both in our validation series and in the TCGA database. Additionally, *SMYD2* expression levels differed significantly between chRCCs and oncocytomas, indicating a potential for discriminating among these 2 RCT subtypes, which frequently display overlapping and confounding morphological features that might impair differential diagnosis, especially in small biopsies. Importantly, mutational analysis data accessible in the COSMIC database (http://cancer.sanger.ac.uk/cosmic)[46] revealed that, in RCC, *SMYD2*, and *SETD3* mutations are exceedingly rare [mutation rate of 0.22% (3/1345) and 0.32% (4/1253), respectively] and were not reported for *NO66*. Thus, it seems unlikely that the differences in gene expression we observed might be due to genetic alterations.

We also found significantly higher expression levels of *SMYD2, SETD3*, and *NO66* in RCTs than in RNTs in the validation series. These results are similar to those observed in the TCGA dataset for *SMYD2* expression, keeping in mind that in our validation series the normal tissue was collected from non-RCT patients, whereas in TCGA database morphologically matched normal renal tissue from RCC patients was analyzed. This difference in normal samples may account for the higher *SETD3* expression in normal tissue than in RCC noted in the TCGA data set. Moreover, among the 3 validated genes, *SMYD2* expression levels also displayed the best diagnostic performance for distinction between RCTs and normal renal tissue, thus increasing the spectrum of scenarios in which it may assist in diagnosis and classification of suspicious renal lesions. When compared to other molecular techniques, FISH was reported to allow the identification of RCT subtypes,[47] specially to distinguish chRCC from oncocytoma,[48] but the overlapping genetic alterations mainly between chRCC and oncocytoma might hamper differential diagnosis.[49] A molecular algorithm based on qPCR gene expression correctly identified the RCC subtype in 83.3% of cases,[50] and CGH allowed for the correct diagnosis in 93.5% of ccRCC, 100% of pRCC, 61.5% of chRCC, and 14.3% of oncocytomas,[51] although in both studies less than 80 cases were analyzed.

Interestingly, *SMYD2* and *SETD3* differential expression at transcript level was also apparent at protein level, as assessed by immunohistochemistry, and a statistically significant correlation between the 2 parameters was found. However, the wide variation in immunoexpression within RCT subtype and across subtypes precludes its use as an ancillary tool for histopathological evaluation. Notwithstanding, owing to the increasing availability and use of molecular techniques in diagnostic pathology, the assessment of mRNA expression in tissue samples or in fine-needle aspirates of suspicious lesions is within the reach of many molecular pathology laboratories.

Besides its potential role as diagnostic biomarkers, we aimed also at characterizing the prognostic value of HMTs and HDMs expression in RCCs. Interestingly, low *SMYD2, SETD3*, and *NO66* expression levels associated with worse disease-specific survival and disease-free survival, in univariate analysis. The prognostic value of *SMYD2* overexpression has been reported in leukemia,[37,38] esophageal squamous cell carcinoma,[37,38] and gastric carcinoma,[39] contrarily to our findings in RCC. Besides differences in tumor model, it should be emphasized that survival analyses in our series were mostly influenced by pRCC and ccRCC, which displayed the lowest expression levels for the 3 validated genes, and that are acknowledged as the most aggressive RCC subtypes. Furthermore, in multivariate analysis assessing the expression level of each gene, pT, histological subtype, and gender, statistical significance was retained for pT and expression level for the 3 enzymes, both for DSS and DFS, with low expression level and pT3 associating with worse prognosis, thus indicating that each gene independently adds prognostic information to pT stage. When combining the expression levels of the 3 genes with pathological stage, occurrence of metastasis during follow-up, histological subtype, and gender, for our series and for the

TCGA dataset separately, Stage III / Stage IV and the development of metastasis associated with shorter DSS in both cohorts. Furthermore, low *SETD3* expression and ccRCC and pRCC (compared to chRCC) subtypes also associated with decreased DSS, but only in the TCGA data set. A shorter DFS was associated with low *SMYD2* expression levels and Stage III / Stage IV in our series, and low NO66 and Stage III / Stage IV in the TCGA dataset. These differences might be due to (i) cohort size, (ii) dissimilar proportions of the 3 histological subtypes in the 2 cohorts, or (iii) differences in period of follow-up. Since the number of events, both cancer-specific deaths and development of metastasis, were scarce in our series, mainly in the ccRCC (subtypes 2 and 4, respectively) and chRCC (subtypes 1 and 2, respectively), survival analysis was not performed separately for each subtype. To overcome this limitation, TCGA data set was further explored. Interestingly, when analyzing each subtype separately in multivariate analysis, *SETD3* was associated with shorter DSS in ccRCC and shorter DSS and DFS in pRCC, underlining the potential clinical value of *SETD3* as a prognostic biomarker.

To the best of our knowledge, only a few studies have addressed the role of *SMYD2, SETD3*, and *NO66* in tumorigenesis, as previously described, although none has included RCTs. The knowledge of the specific role of *SMYD2, SETD3*, and *NO66* in renal carcinogenesis, uncovering the participation of these enzymes in the neoplastic transformation of renal epithelium and in RCC progression, might additionally provide a stronger biological rational for its use as clinically useful diagnostic and prognostic biomarkers.

Patients and Methods

Patients and sample collection

A total of 160 RCTs, comprising ccRCCs, pRCCs, chRCCs, and oncocytomas (40 cases of each type), were prospectively collected from patients consecutively diagnosed and submitted to nephrectomy at the Portuguese Oncology Institute – Porto, between 2001 and 2014. As controls, 10 renal normal tissue (RNT) samples were collected from morphologic normal kidneys of patients subjected to nephrectomy due to upper urinary tract urothelial carcinoma. All specimens were immediately frozen after surgery and stored at −80°C. Sampling of more than 70% of malignant cell was confirmed by 2 slides stained with hematoxylin and eosin (H&E) taken before and after frozen section collection for RNA extraction. Routine histological slides from formalin-fixed paraffin-embedded (FFPE) tissue of the same surgical specimens were assessed for diagnosis, TNM staging, and Fuhrman grading. Relevant clinical data was also collected from clinical charts. This study was approved by the Institutional Review Board of Portuguese Oncology Institute – Porto [Comissão de Ética para a Saúde-(CES-IPOFG-EPE 518/10)].

RNA extraction

Samples were suspended in TRIzol® reagent (Invitrogen™, Cat.#15596018) and chloroform (Merk Millipore, Cat. #MCX10601) was added to the lysed cells. Total RNA was purified using the Ambion® PureLink RNA Mini Kit (Invitrogen™, Cat.#12183025), according to manufacturer recommendations. RNA concentrations and purity ratios were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and RNA quality was verified by electrophoresis.

Screening of histone methyltransferases and demethylases

A total of 10 RCT (5 oncocytomas and 5 chRCCs) and 5 RNT samples were treated with Ambion® TURBO DNA-free™ kit (Invitrogen, Cat.#1907) to remove any DNA contamination, and then 1 μg of total RNA was reversely transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems®, Cat.#4368814) according to manufacturer instructions. Expression of histone methylation enzymes (58 HMTs and 29 HDMs), was assessed by RT-qPCR using custom made TaqMan® Array 96-Well expression Plates (Applied Biosystems®, Cat.#4391528) in an ABI-7500 Real-Time PCR system (Applied Biosystems®, Cat.#4351105).

Each gene was run in triplicate and the amount of mRNA was normalized to Glucuronidase β (*GUS*β) and Human 18S rRNA (*18S*) reference genes. Comparative CT method was used to determine the fold-difference in gene expression between RNT and RCT, as well as between chRCC and oncocytoma. Genes that reached statistically significant differences in expression levels between these groups, and displayed higher or lower expression levels in RCT vs. RNT or in chRCC vs. oncocytoma, were selected for further analysis.

Validation of selected enzymes

Candidate genes' mRNA levels were evaluated in a larger series of 160 RCTs, including 40 ccRCCs, 40 pRCCs, 40 chRCCs, 40 oncocytomas, and 10 RNTs. A total of 300ng was reversely transcribed and amplified using TransPlex® Whole Transcriptome Amplification Kit (Sigma-Aldrich®, Cat. #WTA1) purified with QIAquick PCR Purification Kit (QIAGEN, Cat.#28106), and mRNA levels were evaluated using TaqMan® Gene Expression Assays [Applied Biosystems®, Cat.# Hs00220210 m1 (*SMYD2*), Hs00260120 m1 (*SETD3*), Hs02743012 s1 (*NO66*), Hs99999908 m1 (*GUSB*), Hs99999901 s1 (*18s*)], according to manufacturer's instructions. For each sample, expression levels were normalized using 2 internal reference genes, *GUS*β and *18S*, according to the formula: target gene relative expression = target gene expression level / [(*GUS*β expression level + *18S* expression level) / 2]. Each plate included multiple non-template controls and serial dilutions of a cDNA Human Reference Total RNA (Agilent Technologies, Cat.#750500) to construct a standard curve.

Immunohistochemistry

A representative slide from 120 RCT cases of the validation cohort (30 cases available from each subtype) was selected and 4 μm sections from FFPE tissue were obtained. Briefly, after deparaffinization antigen retrieval was performed by heating (20 min) in an antigen unmasking solution (Vector Laboratories, Cat.#H3300), endogenous peroxidase activity was neutralized with

0.6% hydrogen peroxide (Merk Millipore, Cat.#107298) for 20 minutes. Then, protein detection was performed using the Novolink™Max Polymer Detection System (Leica Biosystems, Cat. #RE7260-K), according to manufacturer instructions. Slides were incubated in a humid chamber with rabbit polyclonal antibodies, specific for SET and MYND domain containing 2 (SMYD2) (Sigma Aldrich®, Cat.#HPA029023) in a 1:250 dilution (4°C, overnight) and specific for SET domain containing 3 (SETD3) (Novus Biologicals®, Cat.#NBP-88416) in a 1:200 dilution (room temperature, 1 hr). For NO66 several primary antibodies were tested but none was found to provide reliable results.

All washings were performed with Tris buffered saline with Tween® 20 (TBS-T) (Sigma-Aldrich®, Cat.#T9039). To unveil antigen-antibody binding reaction, slides were incubated for 7 minutes, in the dark, in a 0.05% (m/v) 3, 3′-diaminobenzidine (DAB) solution (Sigma-Aldrich®, Cat.#D7304) in phosphate-buffered saline (PBS) (Biochrom Ltd., Cat.#L1835). Then, slides were counterstained with hematoxylin (Merck Millipore, Cat. #105174), dehydrated and diaphanized.

Slides were evaluated by 2 pathologists for SMYD2 and SETD3 immunoexpression and classified using a semi-quantitative scale for both staining intensity (0 – no staining; 1 – intensity lower than normal kidney; 2 – intensity equal to normal kidney; 3 – intensity higher than normal kidney) and percentage of positive cells (0 – $<$ 10%; 1 – 10-33%; 2 – 33-67%; 3 – $>$67%), in each tumor. Staining intensity and percentage of positive cell scores were combined (Score S $=$ staining intensity x percentage of positive cells) to assign a composite score in each tumor, which was then stratified into low expression (S $<$ 4) and high expression (S $\geq$ 4) groups, which basically correspond to RCTs with less than 33% stained cells or staining intensity lower than normal kidney, and RCTs with at least 33% stained cells with an intensity equal or higher than normal kidney.

TCGA dataset analysis in renal cell carcinoma patients

The Cancer Genome Atlas (TCGA) was used to obtain data on *SMYD2, SETD3* and *NO66* expression and clinical information, when available, from renal cell carcinoma patients and matched normal tissue samples.[52] All expression data from samples hybridized by the University of North Carolina, Lineberger Comprehensive Cancer Center, using Illumina HiSeq 2000 RNA Sequencing version 2 analysis, were downloaded from TCGA data matrix (http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp).[52] This data set included 533 ccRCC and 72 matched normal patient samples, 290 pRCC and 32 matched normal patient samples, and 66 chRCC and 25 matched normal patient samples. To prevent duplicates, when there was more than one portion per patient, median values were used. The provided value was pre-processed and normalized according to "level 3" specifications of TCGA (see http://cancergenome.nih.gov/dataportal/ for details). Clinical data of each patient was provided by the Biospecimen Core Resources (BCRs). This data is available for download through TCGA data matrix (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm).[52]

Statistical analysis

Chi-square, Fisher's exact test and, non-parametric tests were used to ascertain the statistical significance of differences among groups of samples, namely Kruskal-Wallis test (KW) for multiple comparisons and Mann-Whitney U test (MW) for pair-wise comparisons, as appropriate. Spearman's test was carried out to ascertain correlations between age and HMTs or HDMs expression levels, as well as between HMTs and HDMs mRNA expression levels.

Receiver operator characteristics (ROC) curves were constructed to assess the diagnostic performance of biomarkers, by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity), and the area under the curve (AUC) was calculated. Sensitivity, specificity, positive and negative predictive values, and accuracy were calculated based on cutoff values based on ROC curve analysis, prioritizing specificity and then sensitivity.

For survival analysis, a 7-year follow-up time was considered, both for disease-specific survival (DSS) and disease free survival (DFS), as patient recruitment occurred during 13 years. Prognostic significance of standard clinicopathological variables (histological subtype, pathological stage, Fuhrman grade, age, gender) and as well as of HMTs and HDMs expression levels, was assessed by constructing disease-specific and disease-free survival curves using the Kaplan-Meier method, with log-rank test and Cox-regression analysis for each variable (univariate test). For this purpose, expression levels of *SMYD2, SETD3*, and *NO66* were classified as low or high based on the 25th percentile expression value of each gene. Similarly, *SMYD2* and *SETD3* immunoreactivity was classified as low or high according to Score S. A Cox-regression model using Forward Stepwise (conditional) test comprising the different variables (multivariate test) was also performed, including the 160 RCC patients, both for disease-specific (DSS) and disease-free (DFS) survival. A similar Cox-regression analysis (univariate and multivariate) was performed for patients from the TCGA dataset, and expression levels were classified as low or high based on the 25th percentile expression value of each gene, too.

Statistical significance was set at $P < 0.05$. Bonferroni's correction was applied for pairwise comparisons following multiple groups' analyses. Statistical analysis was performed using SPSS software for Windows, version 22.0 (IBM-SPSS Inc.), and graphs were built using GraphPad Prism 6.0 software for Windows (GraphPad Software Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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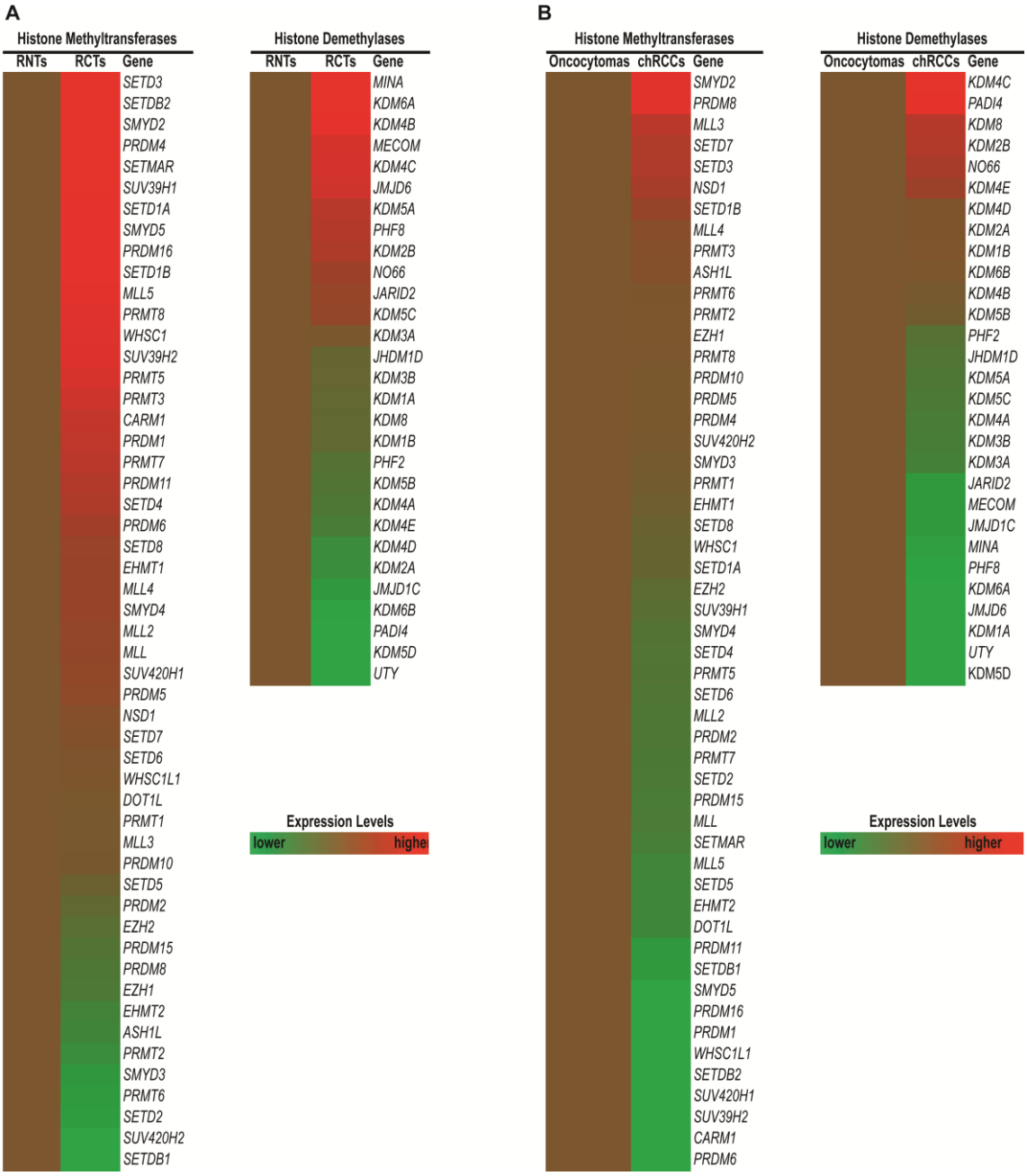
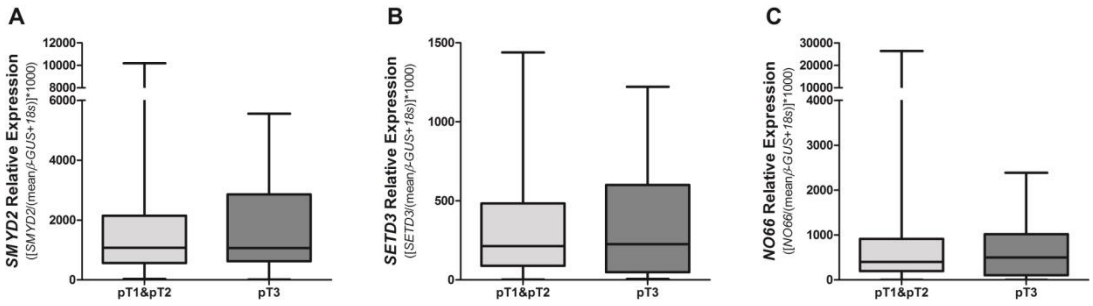
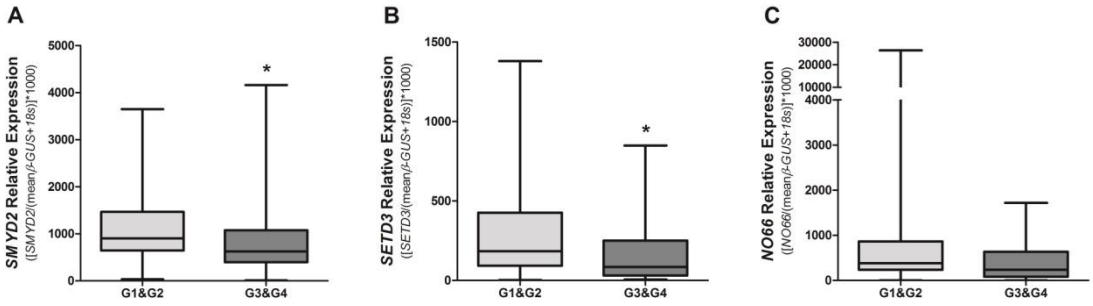
SUPPLEMENTARY DATA



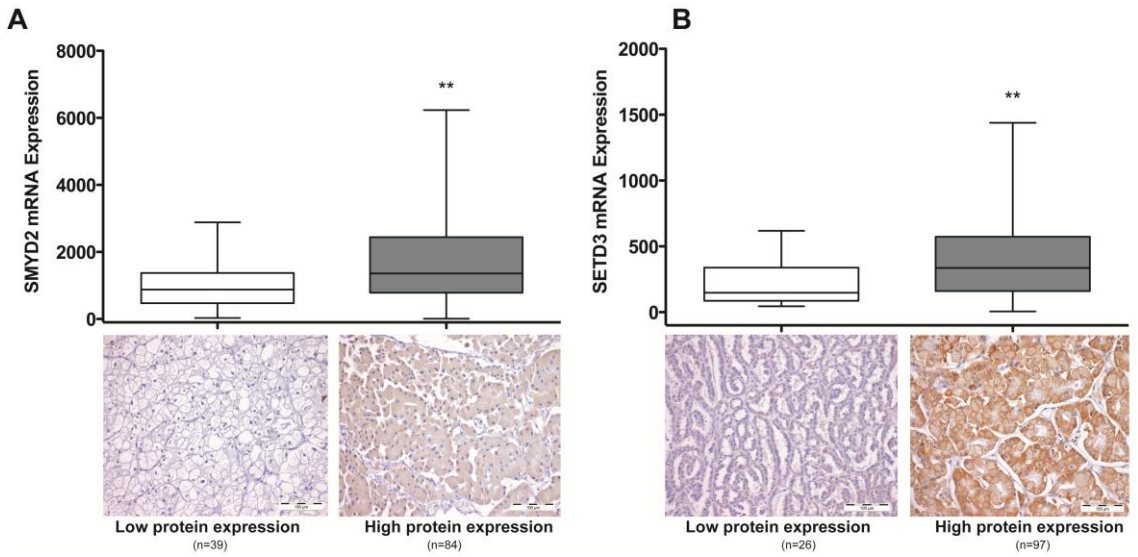
Supplementary Figure 1. Expression levels of 52 HMTs and 29 HDMs in renal tissues. Gene expression was calculated using comparative CT method and the ΔΔCT using*18S* and *GUSβ* as endogenous control genes and RNTs as control group. Each sample was run in triplicate. The results presented correspond to median value of each group. (A) Five renal normal tissues (RNTs) and ten renal cell tumors (RCTs). (B) Five oncocytomas and five chromophobe renal cell carcinomas (chRCCs).



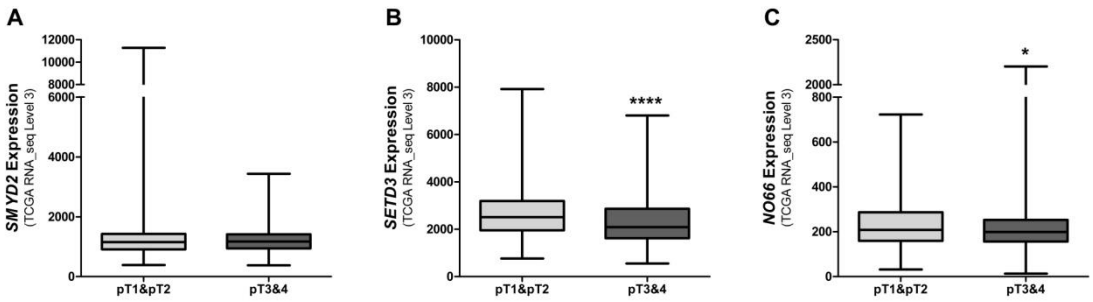
Supplementary Figure 2. Distribution of *SMYD2* (A), *SETD3* (B) and *NO66* (C) expression levels in kidney confined RCC (pT1/pT2) and locally invasive RCC (pT3).



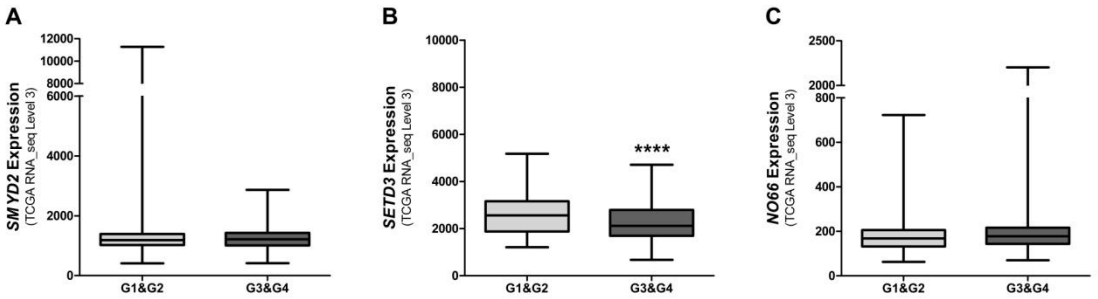
Supplementary Figure 3. Distribution of *SMYD2* (A), *SETD3* (B) and *NO66* (C) expression levels in low Fuhrman grade (G1/G2) and high Fuhrman grade (G3/G4) clear cell and papillary Renal Cell Carcinoma.



Supplementary Figure 4. Distribution of *SMYD2* (A) and *SETD3* (B) mRNA expression levels in RCTs according to low and high protein expression assessed by immunohistochemistry (IHC). Representative IHC images for each group of protein expression. (** p<0.01). IHC combined score (S) = staining intensity x percentage of positive cells; low protein expression (S<4): tumors with less than 33% of stained cells or staining intensity lower than normal kidney; high protein expression (S≥4): tumors with at least 33% of cells stained with an intensity equal or higher than normal kidney.



Supplementary Figure 5. Distribution of *SMYD2* (A), *SETD3* (B) and *NO66* (C) expression levels in kidney confined RCC (pT1/pT2) and locally invasive RCC (pT3) in TCGA dataset (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm**.** Accessed 2015).



Supplementary Figure 6. Distribution of *SMYD2* (A), *SETD3* (B) and *NO66* (C) expression levels in low Fuhrman grade (G1/G2) and high Fuhrman grade (G3/G4) clear cell and papillary Renal Cell Carcinoma patients' samples from the TCGA dataset (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm**.** Accessed 2015).

Supplementary Table 1. Comparison of *SMYD2*, *SETD3* and *NO66* expression levels in kidney confined RCC (pT1/pT2) and locally invasive RCC (pT3).

Expression [median (range)]	pT1/pT2 (n=91)	pT3 (n=29)	Mann-Whitney test
SMYD2	1074 (32-10190)	1066 (11-5550)	p=0.67
SETD3	214 (1.8-1439)	226 (6-1221)	p=0.73
NO66	399 (3-26363)	499 (2-2386)	p=0.86

Supplementary Table 2. Comparison of *SMYD2*, *SETD3* and *NO66* expression levels in low Fuhrman grade (G1/G2) and high Fuhrman grade (G3/G4) clear cell and papillary renal cell carcinoma.

Expression [median (range)]	G1/G2 (n=35)	G3/G4 (n=45)	Mann-Whitney test
SMYD2	903 (32-3649)	625 (11-4159)	p=0.045
SETD3	184 (1.8-1380)	85 (6-849)	p=0.021
NO66	381 (3-26363)	236 (2-1719)	p=0.05

Supplementary Table 3. Pathological stage (pT) and gene (*SMYD2*, *SETD3*, *NO66*) expression level as prognostic factors for renal cell carcinoma obtained by multivariate Cox regression analysis.

Prognostic Factor	Multivariate Analysis		
	Hazard Ratio (HR)	95% CI for HR	Cox regression p value
Disease Specific Survival			
pT §	8.408	2.169-32.590	0.002
SMYD2 expression level *	4.777	1.344-16.973	0.016
Subtype, Gender	-	-	N.S.
pT §	6.340	1.622-24.778	0.008
SETD3 expression level *	5.491	1.403-21.495	0.014
Subtype, Gender	-	-	N.S.
pT §	7.277	1.876-28.224	0.004
NO66 expression level *	3.978	1.118-14.159	0.033
Subtype, Gender	-	-	N.S.
Disease Free Survival			
pT §	3.939	1.264-12.271	0.018
SMYD2 expression level *	10.912	2.939-40.511	<0.001
Subtype, Gender	-	-	N.S.
pT §			0.092
SETD3 expression level *	9.778	2.643-36.172	0.001
Subtype, Gender	-	-	N.S.
pT §			0.050
NO66 expression level *	6.220	1.868-20.709	0.003
Subtype, Gender	-	-	N.S.

CI: Confidence Interval, N.S.: not-significant (p>0.05). § Stage I / Stage II *vs* Stage III / Stage IV; reference group: pT1/pT2. * Reference group: high expression level.

Supplementary Table 4. Clinical and pathological data of patients included in TCGA dataset (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm. Accessed 2015).

	Normal Matched	Tumor
Number of patients, n	129	889
Age, median (range)	61 (28-90)	60 (17-90)
Gender, n (%)		
Male	87 (67.44%)	579 (66.94%)
Female	42 (32.56%)	286 (33.06%)
Histological subtype, n (%)		
Clear cell RCC	72 (55.81%) *	533 (59.96%)
Papillary RCC	32 (24.81%) *	290 (32.62%)
Chromophobe RCC	25 (19.38%) *	66 (7.42%)
Pathological stage, n (%)		
pT1	52 (40.31%) *	468 (54.61%)
pT2	25 (19.38%) *	123 (14.35%)
pT3	48 (37.21%) *	251 (29.29%)
pT4	4 (3.10%) *	15 (1.75%)
Fuhrman grade, n (%) ¥		
1	1 (1.39%) *	13 (2.50%)
2	28 (38.89%) *	228 (43.76%)
3	28 (28.89%) *	205 (39.35%)
4	15 (20.83%) *	75 (14.40%)

RCC, Renal Cell Carcinoma. * For normal renal tissue, histological subtype, pathological stage and Fuhrman grade of the matched RCC is depicted. ¥ Data available only for clear cell Renal Cell Carcinoma.

Supplementary Table 5. Prognostic factors for TCGA dataset (http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm. Accessed 2014) for each RCC subtype by Cox regression analysis.

Prognostic Factor	Univariate Analysis			Multivariate Analysis		
	HR	95% CI for HR	p value¥	HR	95% CI for HR	p value¥
ccRCC						
Disease Specific Survival						
Pathological Stage§	**4.35**	**3.14-6.02**	**<0.0001**	**2.74**	**1.86-4.04**	**<0.0001**
Metastasis(during follow-up)φ	**4.70**	**3.43-6.44**	**<0.0001**	**2.37**	**1.63-3.43**	**<0.0001**
SMYD2 expression level*	**1.53**	**1.05-2.21**	**0.027**			0.067
SETD3 expression level*	**2.16**	**1.59-2.95**	**<0.0001**	**1.77**	**1.29-2.42**	**<0.0001**
NO66 expression level*			0.389			0.749
GenderΨ			0.838			0.460
Disease Free Survival						
Pathological Stage§	**8.97**	**1.39-57.70**	**0.021**	**8.97**	**1.39-57.70**	**0.021**
SMYD2 expression level*			0.212			0.305
SETD3 expression level*			0.796			0.585
NO66 expression level*			0.185			0.171
GenderΨ			0.230			0.355
pRCC						
Disease Specific Survival						
Pathological Stage§	**5.26**	**2.57-10.76**	**<0.0001**	**3.57**	**1.61-7.95**	**0.002**
Metastasis(during follow-up)φ	**93.65**	**27.68-316.92**	**<0.0001**	**43.99**	**11.46-168.87**	**<0.0001**
SMYD2 expression level*			0.912			0.941
SETD3 expression level*	**2.84**	**1.43-5.63**	**0.003**	**2.11**	**1.00-4.45**	**0.051**
NO66 expression level*	**8.00**	**2.66-24.05**	**<0.0001**	**6.49**	**2.00-21.11**	**0.002**
GenderΨ			0.230			0.097
Disease Free Survival						
Pathological Stage§	**7.78**	**2.34-25.89**	**0.001**	**7.25**	**2.19-24.03**	**0.001**
SMYD2 expression level*			0.777			0.967
SETD3 expression level*	**4.74**	**1.44-15.57**	**0.010**	**4.64**	**1.39-15.51**	**0.013**
NO66 expression level*	**6.98**	**1.48-32.96**	**0.014**			0.227
GenderΨ			0.095			0.381
chRCC [a]						
Disease Specific Survival						
Pathological Stage§	**6.69**	**1.73-25.95**	**0.006**	**11.86**	**1.38-101.81**	**0.024**
Metastasis(during follow-up)φ	**20.50**	**1.86-226.04**	**0.014**			0.052
SMYD2 expression level*			0.450			0.641
SETD3 expression level*	**7.22**	**1.37-37.94**	**0.020**			0.303
NO66 expression level*			0.484			0.600
GenderΨ			0.396			0.171

HR: Hazard Ratio; CI: Confidence Interval, n.s.: not significant. ¥Cox regression p value; significant when p<0.05. §Stage I / Stage II *vs* Stage III / Stage IV; reference group: Stage I / Stage II. *Reference group: high expression level. φReference group: Absence of metastasis. ΨReference group: Male. [a] Disease Free survival was not performed for chRCC due to insufficient available data.

Supplementary Table 6. *SMYD2*, *SETD3* and *NO66* expression in "GEO DataSet" (http://www.ncbi.nlm.nih.gov/gds. Accessed 2015), comprising eighteen patients, nine with oncocytoma and nine with chromophobe Renal Cell Carcinoma. Mann-Whitney was used for comparison between oncocytoma and chRCC.

Expression [median (range)]	Oncocytoma (n=9)	chRCC (n=9)	Mann-Whitney test
SMYD2 [ID REF: 212921_at]	0.29 (0.27- 0.47)	0.44 (0.28 – 0.85)	0.031
SMYD2 [ID REF: 212922_s_at]	24.9 (5.4 - 9373)	23.5 (10.5 - 8655)	1
SETD3 [ID REF: 229940_at]	1033 (0.76 - 1192)	1205 (1.3 - 2612)	0.011
NO66 [ID REF: 219526_at]	2889 (2234 - 3344)	3833 (2657 - 7663)	0.008

5.3. *SETDB2* AND *RIOX2* ARE DIFFERENTIALLY EXPRESSED AMONG RENAL CELL TUMOR SUBTYPES, ASSOCIATING WITH PROGNOSIS AND METASTIZATION

SETDB2 and *RIOX2* are differentially expressed among renal cell tumor subtypes, associating with prognosis and metastization

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Abstract

Increasing detection of small renal masses by imaging techniques, entails the need for accurate discrimination between benign and malignant renal cell tumors (RCTs) as well as among malignant RCTs, owing to differential risk of progression through metastization. Although histone methylation has been implicated in renal tumorigenesis, its potential as biomarker for renal cell carcinoma (RCC) progression remains largely unexplored. Thus, we aimed to characterize the differential expression of histone methyltransferases (HMTs) and histone demethylases (HDMs) in renal cell tumors (RCTs) to assess their potential as metastasis biomarkers. We found that *SETDB2* and *RIOX2* (encoding for an HMT and an HDM, respectively) expression levels was significantly altered in RCTs, and were further selected for validation by

quantitative RT-PCR in 160 RCTs. Moreover, *SETDB2*, *RIOX2* and three genes encoding for enzymes involved in histone methylation (*NO66*, *SETD3* and *SMYD2)*, previously reported by our group, were quantified (RT-PCR) in an independent series of 62 clear cell renal cell carcinoma (ccRCC) to assess its potential role in ccRCC metastasis development. Additional validation was performed using TCGA dataset. *SETDB2* and *RIOX2* transcripts were found overexpressed in RCTs compared to renal normal tissues (RNTs), in oncocytomas vs. RCCs, with ccRCC and papillary renal cell carcinoma (pRCC) displaying the lowest levels. Low *SETDB2* expression levels and higher stage independently predicted shorter disease-free survival. In our 62 ccRCC cohort, significantly higher *RIOX2*, but not *SETDB2*, expression levels were depicted in cases that developed metastasis during follow-up. These findings were not apparent in TCGA dataset. We concluded that *SETDB2* and *RIOX2* might be involved in renal tumorigenesis and RCC progression, especially in metastatic spread. Moreover, *SETDB2* expression levels might independently discriminate among RCC subgroups with distinct outcome, whereas higher *RIOX2* transcript levels might identify ccRCC cases with more propensity to endure metastatic dissemination.

Keywords: Kidney cancer, renal cell tumor, renal cell carcinoma, histone methyltransferase, *SETDB2*, *RIOX2*, biomarker, prognosis, metastasis

Running title: SETDB2 and RIOX2 deregulation associates with renal cell tumor metastization.

Introduction

Kidney cancer incidence is increasing worldwide, with 62,700 new cases and 14,240 deaths estimated for 2016 [1]. Increasing incidence has been attributed to the rising number of incidental small renal tumours diagnosed due to widespread use of imaging techniques, as well as to ageing, obesity and smoking, which are known risk factors for the development of kidney cancer [2]. Increased detection of small renal masses emphasises the need for accurate discrimination not only between benign and malignant RCTs, but also among malignant RCTs subtypes. Indeed, renal cell carcinomas (RCCs) more likely to behave aggressively and to develop metastases should be clearly distinguished from those that will probably have a more indolent growth and might be managed more conservatively [3, 4]. Among RCCs, the most frequent are clear cell renal cell carcinoma (ccRCC), papillary renal cell carcinoma (pRCC) and chromophobe renal cell carcinoma (chRCC). Whereas ccRCC is the histotype that more frequently develops metastases, pRCC is more frequently multifocal and chRCC is mostly an indolent cancer that rarely develops metastases, although its differential diagnosis with oncocytoma, a benign tumour, might be challenging[5].

Metastasis is the foremost cause of cancer-related mortality, despite improvements in diagnosis, surgical techniques, patient care and adjuvant therapies. Biologic heterogeneity of tumour cells, as well as differences in metastatic tumour microenvironment at different sites may influence response to therapy [6]. Thus, understanding pathogenesis of metastases at cellular and molecular level has become a major goal in cancer

research [6, 7]. Indeed, management of metastatic renal cell carcinoma (mRCC) remains a major clinical challenge. Although median survival of patients with mRCC has been increasing due to therapeutic advances, specifically in antiangiogenic drugs and tyrosine-kinase inhibitors (from approximately 12 months with cytokine therapy to more than 26 months with VEGF inhibitors therapy) [8], 5-year survival for advanced kidney cancer was only 11.7% in the period 2007-2013 [9]. Albeit the proportion of patients with mRCC at diagnosis has declined, due to improved imaging techniques as well as more intense screening and incidental case ascertainment, a sizeable number of small RCCs (< 4cm diameter) may present renal capsule invasion, tumour thrombus or lymphatic and distant metastasis [3, 4], and their identification constitutes a major challenge.

Altered epigenetic homeostasis has been implicated in tumorigenesis and epigenetic-based biomarkers may assist in diagnosis, prognostication and prediction of response to targeted therapy [10]. Histone modifications and chromatin modulators, in particular, have been shown to play an important role in cancer progression [11]. In RCC, certain histone modifications associate with progression-free survival and correlate with pathological characteristics of tumours [12]. In addition, defects in epigenetic enzymes, involved in chromatin remodelling and packaging, have been implicated in development of RCTs, reflecting the role of these mechanism in renal tumorigenesis [13]. Herein, we aimed to investigate the potential of HMTs and HDMs expression as biomarkers of metastatic progression in RCC, using two independent RCTs cohorts, complemented with external

validation in TCGA dataset. We selected *SETDB2* (an HMT) and *RIOX2* (an HDM), based on an extended characterization of histone methyltransferases in RCTs, previously reported by our group [14]. Additionally, we also tested *SMYD2* and *SETD3* (both HMT), as well as *NO66* (HDM), previously evaluated in our first RCT cohort and TCGA dataset [14], in the second cohort comprising ccRCC with indolent (non-metastatic) and aggressive (metastatic) disease.

Results

Validation of RIOX2 and SETDB2 expression in RCTs

RIOX2 and *SETDB2* expression levels were assessed by quantitative RT-PCR in a series of 160 RCTs and 13 RNTs. The results were fully concordant with those of the TaqMan® Array as both genes were significantly overexpressed in RCTs compared to RNTs ($p<0.0001$ for *SETDB2* and $p<0.05$ for *RIOX2*; (Figures 1A and 1B). Moreover, *RIOX2* and *SETDB2* expression levels differed significantly between benign and malignant RCTs (Figures 1C and 1D), and among the four RCT subtypes (Table 1). Oncocytomas displayed the highest *SETDB2* and *RIOX2* expression levels, followed by chRCC (Figures 1E and 1F and Table 1).

Table 1. Comparison of SETDB2 and RIOX2 expression among renal normal tissue (RNT), renal cell tumors (RCT), renal cell carcinoma (RCC) and RCT histotypes. For histotype pairwise comparison, the values were statistically significant when $p<0.0125$ (Bonferroni's correction).

	SETDB2 (p value)	***RIOX2*** (p value)
RNT vs RCT	**<0.001**	**<0.05**
RNT vs RCC	**0.001**	0.444
Oncocytoma vs RCC	**<0.001**	**<0.001**
ccRCC vs pRCC	0.392	0.658
ccRCC vs chRCC	**<0.001**	**<0.001**
ccRCC vs oncocytoma	**<0.001**	**<0.001**
pRCC vs chRCC	**<0.001**	**<0.001**
pRCC vs oncocytoma	**<0.001**	**<0.001**
chRCC vs oncocytoma	**<0.001**	0.131

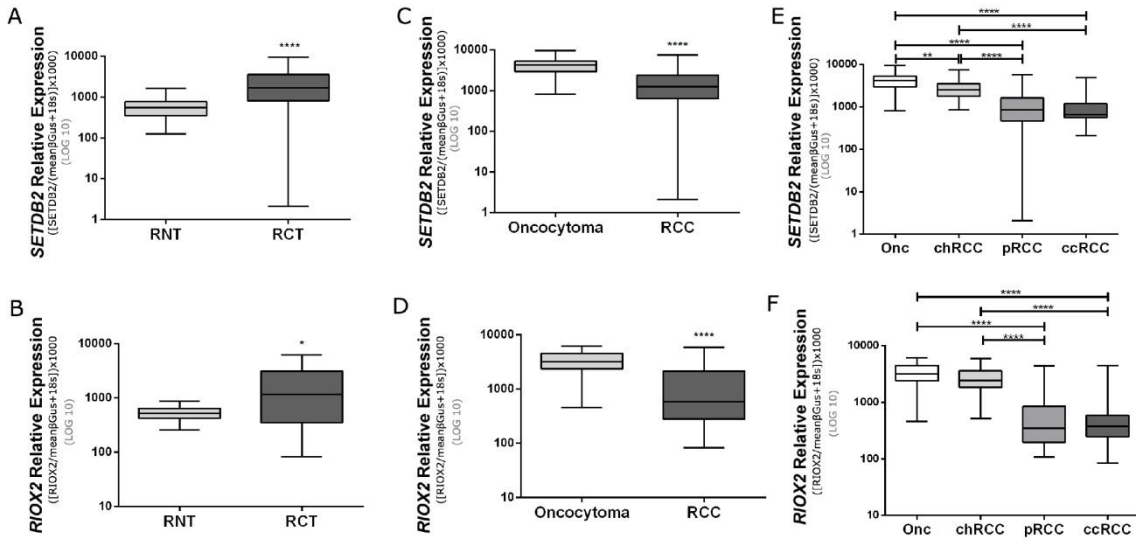

Figure 1. Expression levels of SETDB2 and RIOX2 in cohort#1. A: SETDB2 expression in renal cell tumors (RCTs) and renal normal tissues (RNTs) B: RIOX2 expression in renal cell tumors (RCTs) and renal normal tissues (RNTs) C: SETDB2 expression in benign tumors (oncocytoma) and malignant tumors (renal cell carcinoma [RCCs]); D: RIOX2 expression in benign tumors (oncocytoma) and malignant tumors (renal cell carcinoma [RCCs]); E: SETDB2 expression in renal cell tumors subtypes; F: RIOX2 expression in renal cell tumors subtypes. (****p<0.0001; **p<0.01; *p<0.05).

Pairwise comparisons demonstrated that *SETDB2* and *RIOX2* expression levels significantly differed between chRCC and both pRCC and ccRCC, and between pRCC and both chRCC and oncocytoma. Furthermore, *SETDB2* transcript levels differed significantly between chRCCs and oncocytomas (Figures 1E and 1F and Table 1).

SETDB2 and RIOX2 expression levels and clinicopathological correlates

No significant differences in gender and age were apparent between patients and controls. In RCCs, no statistically significant associations were disclosed between *SETDB2* and *RIOX2* expression levels and Fuhrman or pathological stage categories. In RCTs, expression levels of both genes were significantly

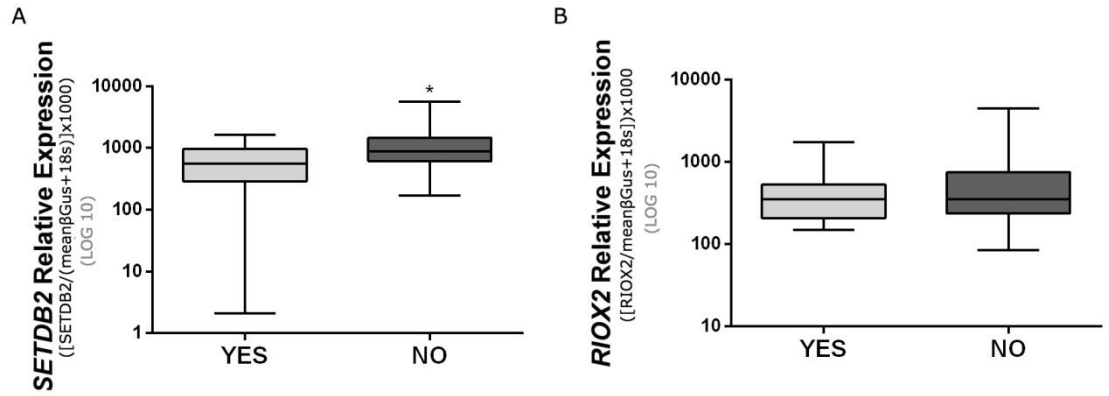

Figure 2. Expression levels of SETDB2 (A) and RIOX2 (B) in clear cell renal cell carcinomas and papillary renal cell carcinomas (cohort#1) with or without metastasis (p<0.05).

higher in females. Moreover, *RIOX2* expression levels significantly associated with patient's age (p=0.015). In ccRCCs and pRCCs, *SETDB2* expression levels were significantly lower in patients that developed metastases (Figures 2A and 2B).

SETDB2 and RIOX2 expression levels as prognostic markers

The median follow-up of RCC patients was 175 months (range: 2-375 months). A total of 15 patients died from RCC during this period. In univariable analysis, higher pathological stage (pT3 or higher) associated with shorter survival whereas gender, age, histological subtype and Fuhrman grade did not disclose any prognostic value within the available follow-up time. Disease-specific survival (DSS) analysis showed that low *SETDB2* and *RIOX2* levels were significantly associated with worse outcome (p<0.01 and p<0.05 respectively; (Figures 3A and 3B). Concerning disease-free survival (DFS) analysis, low *SETDB2*

levels significantly associated with shorter time to disease progression ($p<0.0001$; Figure 3C). The same trend was observed for *RIOX2*, but statistical significance was not reached ($p=0.055$; Figure 3D). In this series, only one case with local recurrence presented distant metastasis before local recurrence developed, thus DFS is equivalent to metastasis-free survival in this case. In multivariable analysis, a final model including *SETDB2* expression levels and pathological stage was predictive of disease-free survival.

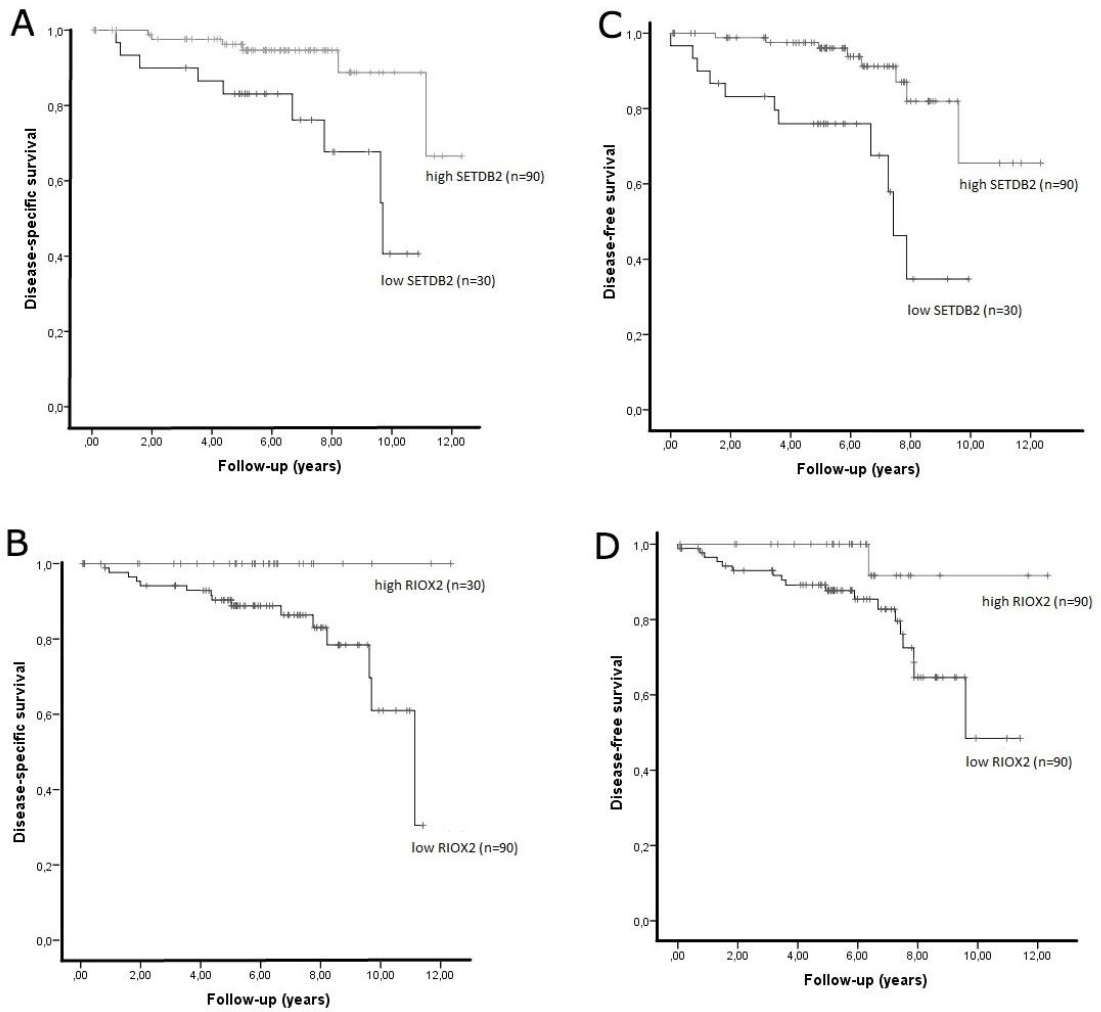

Figure 3. Kaplan-Meier estimated disease-specific survival curves and disease-free survival curves for SETDB2 (respectively A and C) and RIOX2 (respectively B and D).

Indeed, higher risk of disease progression was depicted for patients with higher pathological stage [HR: 3.03 (1.16-7.80), p=0.024] and lower *SETDB2* expression levels [HR: 5.11 (1.72-15.24), p=0.003].

RIOX2, SETDB2, SETD3, SMYD2 and NO66 expression and risk of metastization in ccRCC

No significant differences were apparent for gender and age (p=0.570 and p=0.402 respectively) between ccRCCs patients which developed metastases and those that did not (cohort #2). Furthermore, no statistically significant associations were disclosed between *SETDB2* and *RIOX2* expression levels and Fuhrman grade or pathological stage in this cohort. In this cohort, expression levels of *SETD3, SMYD2* and *NO66*, which we have previously found to associate with shorter disease-specific and disease-free survival [14], did not significantly differ between the two groups of ccRCC patients (Figures 4C, 4D and 4E). Concerning *RIOX2* and *SETDB2* expression levels, only the former differed significantly between metastasized and non-metastasized ccRCCs (Figure 4B).

RIOX2 and SETDB2 expression in RCC Patients from TCGA Dataset

In TCGA dataset, significantly lower *RIOX2* expression levels were found in RCC compared to RNT, contrarily to our results. Nevertheless, among RCCs, pairwise comparisons showed that *RIOX2* expression levels were significantly higher in chRCCs compared to ccRCCs and pRCCs, paralleling our findings. In ccRCCs from TCGA

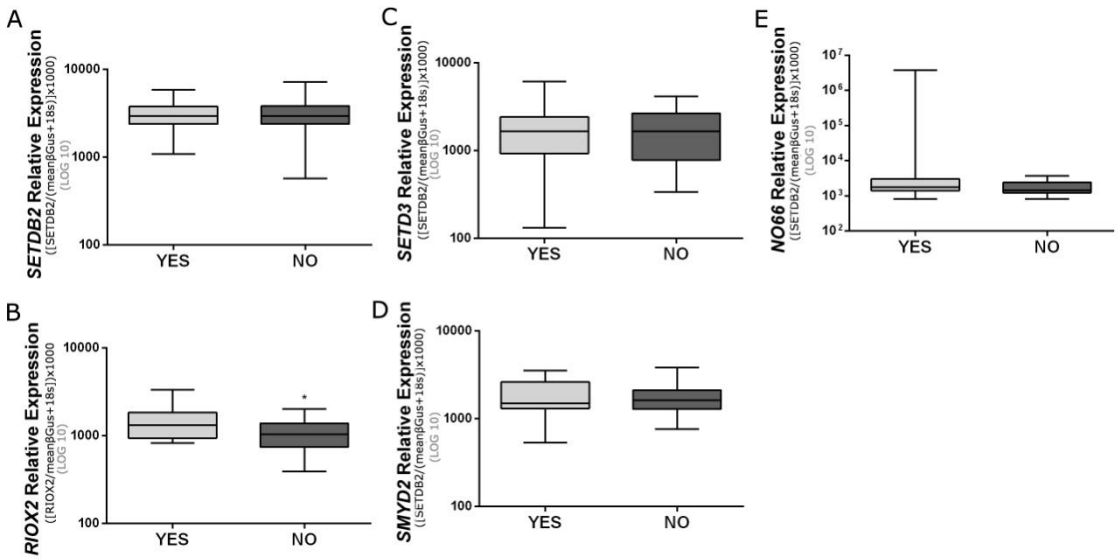

Figure 4. Expression levels of SETDB2 (A), RIOX2 (B), SETD3 (C), SMYD2 (D) and NO66 (E) in clear cell renal cell carcinomas (cohort#2) with or without metastasis ($p<0.05$).

database, no statistically significant difference was disclosed for *RIOX2* expression levels between the group of patients that developed metastases and those that did not.

Concerning *SETDB2*, lower expression levels were depicted in RCC compared to RNT, as well. In line with our results, however, pairwise comparisons demonstrated that *SETDB2* expression levels were significantly higher in chRCCs compared to ccRCCs and pRCCs, and expression levels significantly differed among subtypes. Considering only ccRCCs from TCGA database, no statistically significant difference was apparent for *SETDB2* expression levels between the group of patients that developed metastases and those that did not, paralleling our results.

Discussion

Due to the widespread use of imaging tests, the frequency of incidentally detected RCTs has significantly increased, consisting mainly of small and early stage tumours. However, as lymph node and distant metastases may occur even in small RCCs [15] and the latter constitute the main cause RCC-related mortality [16], there is an unmet need for biomarkers capable of accurately discriminate tumours that will metastasize from those that will not, especially among pT1, which are the most amenable to nephron-sparing surgery. Because epigenetic-based biomarkers may assist in diagnosis, prognostication and prediction of response to targeted therapy [10], we hypothesized that histone modifications and chromatin modulators [11] might aid in the identification of RCCs more prone to recur and metastasize. Indeed, previous reports have shown that, in RCC, histone modifications are associated with pathological features and DFS [12], and defects in chromatin remodelers and chromatin packaging are implicated in RCT development [13].

In this study, we focused mostly on *RIOX2* and *SETDB2* expression levels as candidate biomarkers for RCC prognostication. Whereas *RIOX2* encodes for an HDM, displaying high transcript or protein levels in RCC and several other cancers, associating with poor prognosis [17-24], *SETDB2* encodes for an HMT involved in leukemogenesis [15] but was not previously associated with solid tumors. These two genes were selected based on previously published array data from our team [14], which were confirmed through analysis of cohort #1 tissue samples. Indeed, both *RIOX 2* and *SETDB2* were found overexpressed in RCTs compared to non-paired normal renal tissues,

suggesting that their deregulation is associated with neoplastic transformation of renal parenchymal cells. Interestingly, renal oncocytomas displayed the highest *RIOX2* and *SETDB2* expression levels, significantly differing from RCCs. This result might be of practical value for distinction between oncocytoma and chRCC, especially the eosinophilic variant, as both histotypes display variable degree of morphological overlap, rendering differential diagnosis problematic, particularly in small core biopsies [25].

Unexpectedly, when TCGA dataset was interrogated for *RIOX2* and *SETDB2* expression in renal tissues, RCCs displayed significantly lower expression levels than normal renal tissues, contrarily to our findings in cohort #1. Nonetheless, the expression ranking among RCCs in TCGA paralleled our findings, with chRCC displaying the highest expression levels, compared to ccRCC and pRCC. This discrepancy might be related with the origin of the "normal renal tissue" analysed. Indeed, whereas in our study RNT samples derived from kidneys not harbouring RCC, those of TCGA dataset were collected from macroscopically normal looking areas of organs involved by RCC. As we have previously shown, these "paired" normal renal tissue samples disclose significant epigenetic alterations, that may precede neoplastic transformation [26]. Thus, the results from cohort #1 and TCGA dataset analysis may not be directly comparable.

A major goal of this study was to ascertain the prognostic value of *RIOX2* and *SETDB2* expression levels in RCCs. Whereas, in univariable analysis, lower *RIOX2* and *SETDB2* expression levels associated with worse DSS, only lower *SETDB2* levels associated with worse DFS. Interestingly, among standard

clinicopathological parameters, only stage reached statistical significance. In multivariable analysis, however, only low *SETDB2* expression and stage retained statistical significance, suggesting that assessment of SETDB2 expression might add relevant prognostic information for the management of RCC patients. Although a previous report has associated higher RIOX2 immunoexpression with shorter DSS in RCC [17], these results are not directly comparable with ours as we did not assess protein expression and the proportion of RCC subtypes also differed. Indeed, in our series, survival analysis results were mostly influenced by ccRCC and pRCC, which displayed the lowest expression levels among RCCs. Moreover, RIOX2 overexpression has been associated with worse prognosis in oesophageal cancer [24] but with favourable outcome in lung cancer [27], emphasizing that the biological and clinical impact of RIOX2 expression is strongly dependent on the primary location and the specific cancer type.

Because DFS was analogous to metastasis-free survival in cohort #1, we looked for differences in *SETDB2* expression levels among tumours with and without metastasis. Interestingly, we found significantly lower *SETDB2* expression in ccRCC and pRCC (the more clinically aggressive histotypes) with metastasis. However, when we attempted to validate these findings is an independent cohort comprising ccRCC with and without metastasis (cohort #2) and in ccRCCs from TCGA dataset, no significant differences were disclosed. Nonetheless, significantly higher *RIOX2* transcript levels were depicted in ccRCC that developed metastasis during follow-up compared to matched ccRCC without metastasis, suggesting that *RIOX2* expression might be a biomarker of progression

(metastization) in ccRCC. Interestingly, high *RIOX2* expression levels have been associated with development of lymphatic or distant metastasis in bile duct, gastric and pancreatic carcinomas [22, 23, 28]. In TCGA dataset, however, no differences in *RIOX2* expression levels were apparent between ccRCCs that developed metastases and those that did not. These discrepancies might be due to differences in follow-up time and enrolment criteria, as we excluded from analysis cases that presented metastasis at diagnosis and only analysed cases in which metastases developed after curative-intent surgical treatment. Although we further evaluated three genes encoding for histone modifying enzymes (*SMYD2*, *SETD3* and *NO66*) previously shown to be associated with worse prognosis in RCC [14] in cohort #2, their expression levels did not significantly differ between ccRCC with and without metastasis.

Although our findings might be limited by the sample size, it should be emphasized that the most frequent histotypes are represented, whereas in many studies only ccRCC cases have been included. Moreover, survival analysis is based on long-term follow-up data, including two patient cohorts and TCGA dataset. Finally, although candidate biomarkers were previously identified in array-based analysis, validation in independent patient's series was undertaken, whereas several previous studies proposing array-based biomarkers for RCC, have not validated them or have just performed validation in limited series of patients [29-32], precluding the assessment of its clinical usefulness.

In conclusion, our results suggest that *SETDB2* and *RIOX2* might be involved in

renal tumorigenesis and RCC progression, especially in metastatic spread. Moreover, *SETDB2* expression levels might independently discriminate among RCC patient subgroups with distinct outcome, whereas higher *RIOX2* transcript levels might identify ccRCC cases with more propensity to endure metastatic dissemination.

Materials and Methods

Patients and Sample Collection

A series of 160 RCTs (cohort #1) comprising 40 cases of each subtype (ccRCC, pRCC, chRCC and oncocytoma) was prospectively collected from patients consecutively diagnosed and submitted to nephrectomy at the Portuguese Oncology Institute of Porto (IPO Porto). As controls, 13 renal normal tissue (RNT) samples were procured from patients submitted to nephro-ureterectomy due to upper urinary tract urothelial carcinoma, not involving the renal parenchyma. All tissues were immediately frozen and stored at -80ºC. Sampling of more than 70% of malignant cells was confirmed using two slides stained with hematoxylin and eosin (H&E) taken before and after frozen section collection for RNA extraction. Relevant clinical data was retrieved from clinical charts.

An independent series of 62 ccRCCc (cohort #2) comprising 31 ccRCCs that have developed metastasis and 31 ccRCCs that did not progress, matched for gender, age, tumor size, grade and stage at diagnosis, was also retrieved from the archives. Tissue samples were prepared as described above.

This study was approved by the ethics committee of IPO Porto (CES-IPOPFG-EPE 518/10).

RNA Extraction

For RNA extraction, samples were suspended in TRIzol® reagent (Invitrogen™, Carlsbad, CA, USA; Cat. #15596018) and chloroform (Merk Milipore, Darmstadt, Germany; Cat.#MCX10601) was added after the cells were lysed. RNA concentrations and purity ratios were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples were then stored at -80ºc.

HMT and HDM selection

Selection of candidate HMTs and HDMs was based on the results of previously reported custom made TaqMan® Array 96-Well expression plates (Applied Biosystems®, Foster City, CA, USA; Cat#4391528) [14]. *SMYD2*, *SETD3* and *NO66* had been previously validated and found to be associated with shorter disease-specific and disease-free survival [14]. Additionally, *SETDB2* and *RIOX2* expression also displayed high fold variation between RCTs and RCCs, as well as between chRCC and oncocytomas, and were thus selected for further validation. Both presented higher expression in RCT compared to RNT, as well as in oncocytomas than in chRCC.

Validation of Selected Enzymes

RIOX2 and *SEDTB2* mRNA expression levels were firstly evaluated in cohort #1. Subsequently, *RIOX2*, *SETDB2*, *SMYD2*, *NO66* and *SETD3* expression was assessed in cohort #2.

For validation in cohort #1, 300ng of mRNA was reverse transcribed and amplified using TransPlex®Whole Transcriptome Amplification Kit (Sigma-Aldrich®, St.Louis, MO, United States) with subsequent purification using

QIAquick PCR Purification Kit (QIAGEN, Germany), according to manufacturer's instructions. *RIOX2* and *SETDB2* mRNA levels were evaluated using TaqMan® Gene Expression Assays [Applied Biosystems®, Foster City, CA, USA; Hs99999908 m1 (*GUSβ*), Hs99999901 s1 (18s), Hs01126272 m1 (*SETDB2*), Hs00262155 m1 (*RIOX2*)] according to manufacturer's instructions. For each sample, expression levels were normalized using two internal reference gene, *GUSβ* and *18s*, according to the formula: target gene relative expression = target gene expression level / ((*GUSβ* expression level + *18s* expression level) / 2). Each plate included multiple non-template controls and serial dilutions of a cDNA Human Reference Total RNA (Agilent Technologies, La Jolla, CA, USA; Cat.#750500) to construct a standard curve.

For validation in cohort #2, 1 µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. *RIOX2*, *SETDB2*, *SMYD2*, *NO66* and *SETD3* mRNA levels were evaluated using TaqMan® Gene Expression Assays [Applied Biosystems®, Foster City, CA, USA; Cat.#4331182 Hs00220210 m1 (*SMYD2*), Hs00260120 m1 (*SETD3*), Hs02743012 s1 (*NO66*), Hs99999908 m1 (*GUSβ*), Hs99999901 s1 (18s), Hs01126272 m1 (*SETDB2*), Hs00262155 m1 (*RIOX2*)] according to manufacturer's instructions. For each sample, expression levels were normalized using two internal reference gene, *GUSβ* and *18s*, according to the formula: target gene relative expression = target gene expression level / ((*GUSβ* expression level + *18s* expression level) / 2). Each plate included multiple non-template

controls and serial dilutions of a cDNA Human Reference Total RNA (Agilent Technologies, La Jolla, CA, USA; Cat.#750500) in order to construct a standard curve.

TCGA Dataset Analysis in pRCC, chRCC and ccRCCs Patients

The Cancer Genome Atlas (TCGA) dataset was interrogated for data on *RIOX2 and SETDB2* expression and clinical information, when available, from ccRCCs, pRCCs and ccRCCs patients. All expression data from samples hybridized at the University of North Carolina, Lineberger Comprehensive Cancer Center, using Illumina HiSeq 2000 RNA Sequencing version 2 analysis, were downloaded from TCGA data matrix (http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp). This dataset included 533 ccRCC, 290 pRCC and 66 chRCC. The provided value was pre-processed and normalized according to "level 3" specifications of TCGA (see http://cancergenome.nih.gov/dataportal/ for details). Biospecimen Core Resources (BCRs) provided the clinical data of each patient. This data is available for download through TCGA data matrix (http://tcga-data.nci.nih.gov/tcga/dataAccesMatrix.htm).

Statistical Analysis

Non-parametric tests were used to ascertain statistical significance of comparisons among groups. Kruskal-Wallis test (KW) was used for comparisons among multiple groups and Mann-Whitney U test (MW) was used for pairwise comparisons. The prognostic significance of clinicopathological variables (age, gender, histological subtype,

pathological stage, Fuhrman grade) and HMTs and HDMs expression levels was assessed by constructing disease-specific and disease-free survival curves using the Kaplan-Meier method with log-rank test (univariable test). The expression levels of *SETDB2* and *RIOX2* were classified as low or high based on the cutoff value of 25th percentile for *SETDB2* expression and 75th percentile for *RIOX2*. A Cox-regression model comprising the different variables (multivariable test) was also constructed. For this analysis, the 120 RCC patients from cohort #1 were included.

Statistical significance was set at $p<0.05$. Bonferroni correction was applied for pairwise comparisons following multiple groups' analyses. Statistical analysis was performed using SPSS software for Windows, version 22.0 (IBM-SPSS Inc., Chicago, IL, USA), and graphs were built using GraphPad Prism 6.0 software for Windows (GraphPad Software Inc., La Jolla, CA, USA).

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Disclosure Statement

None of the authors have any conflict of interest to declare.

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CHAPTER 6

GENERAL DISCUSSION

6.1. GENERAL DISCUSSION

The last years were fertile in novel data on genetic and epigenetic alterations in renal cell tumors, and especially in integrative, multi-level approaches to data interpretation, fueled by TCGA project [1-4], but not limited to it [5-8]. These approaches allowed for a more comprehensive picture of renal carcinogenesis, reflecting its complexity and corroborating the relevance of epigenetic alterations, mostly histone modulators' alterations [9-11]. Other epigenetic alterations have been also recurrently reported in renal cell carcinomas, including promoter methylation [12-18] and microRNAs deregulation [19-22]. Additional description of distinct epigenetic alterations in different histotypes of renal cell tumors reflects the potential of DNA methyation [23, 24] and miRNA profile [25-27] as promising diagnostic biomarkers.

However, most of the genome-wide approaches that identified these differentially methylated genes were usually not followed by extensive validation in independent series [17, 23, 24] using more sensitive methodologies. Moreover, most of the first discovery series were predominantly or exclusively composed of clear cell renal cell carcinoma [12-17, 19-22], the most frequent renal cell carcinoma subtype, although this trend has been changing [6, 28, 29].

In the scope of this Thesis, quantitative methylation specific polymerase chain reaction (QMSP) was used to assess methylation levels of genes reported to be differentially methylated among renal cell tumor subtypes [23, 24, 30]. QMSP is a sensitive and robust technique, that could be performed in most molecular pathology labs, suitable to study DNA methylation of specific regions [31]. Thus, a panel based on the methylation of specific gene regions could be an economically viable and efficient

methodology to detect specific methylation profiles associated with renal malignancy and/or specific RCT histotypes, with the additional advantages that, as DNA is more stable than RNA, assays detecting DNA alterations are often more robust; DNA methylation is a more homogeneous target than gene mutations; and DNA methylation is an early event in renal tumorigenesis [31-33].

A two-gene promoter methylation panel (*OXR1*&*MST1R*) that distinguishes normal kidney from renal cell tumors, and clear cell renal cell carcinoma from other renal cell tumors with high sensitivity and specificity, was here described. Thus, it might be a useful diagnostic biomarker for clear cell renal cell carcinoma. Its potential would be probably greater as ancillary tool for renal mass biopsy examination, considering the limitations that most methylation-based diagnostic biomarkers present when assessed in biological fluids [34-36]. The diagnostic performance of this panel in detection of malignancy and clear cell renal cell carcinoma was better than most similar panels reported thus far [37]. Although not directly comparable due to distinct methodology, this methylation panel results are globally equivalent to methylation array-based assay panels, which are more demanding [38, 39]. For the identification of oncocytoma, a miRNA panel comprising miRNA-141 and miRNA-200b proved to be more sensitive and specific than the referred methylation panel, in the same cohort of cases [40], suggesting that distinct types of alterations might be more suitable to identify distinct RCT subtypes. Indeed, it is not surprising that methylation-based biomarkers display lower sensitivity and specificity in the distinction between chromophobe renal cell carcinoma and oncocytoma, as methylation array studies reported methylation rates usually not higher than 35% in chromophobe renal cell carcinoma and oncocytoma [24], and globally a lower number of genes were found to be methylated in

chromophobe renal cell carcinoma and oncocytoma compared to clear cell renal cell carcinoma [3, 6, 24]. Conversely, higher promoter methylation frequency correlated with higher stage and grade tumors in the TGGA dataset [1], and therefore is not surprising that grade 3&4 clear cell renal cell carcinomas in our cohort presented higher OXR1 methylation levels.

We also explored the biological and functional effect of promoter methylation on *MST1R* gene expression in kidney tumors. MST1R is a C-MET proto-oncogene family receptor tyrosine kinase, involved in a pathway frequently altered in renal cell carcinoma, especially papillary but also clear cell renal cell carcinoma [1, 2, 4]. *MST1R* promoter methylation pattern was described to be involved in distinct transcript variant expression [41]. This might parallel the increasing importance recognized for the methylation pattern of specific gene transcription regulation [42, 43], but at the promoter level. Although we could associate promoter methylation pattern with the predicted mRNA expression pattern only in clear cell carcinoma cell lines, we purpose that in *in vivo* renal tumors, additional factors might be involved in the regulation of variant expression. However, *MST1R* expression was associated with prognosis, most probably reflecting its relation to a frequently altered cellular pathway in renal cell carcinoma [1, 2, 4].

Histone methyltransferase and histone demethylase expression was also approached to distinguish between chromophobe renal cell carcinoma and oncocytoma, by using a PCR-based array as screening test. This revealed that most histone methyltransferases and demethylases presented altered expression between renal normal tissue and renal cell tumors, and between oncocytoma and chromophobe renal cell carcinoma, probably related to the role of histone alterations in renal tumorigenesis.

We validated five of the most differentially expressed enzymes – *SMYD2*, *SETD3*, *NO66*, *SETDB2* and *RIOX2* – in a large series (cohort#1) of 160 renal cell tumors, with concordant results for chromophobe renal cell carcinoma and oncocytoma. Validation results also revealed similar expression profiles for clear cell and papillary renal cell carcinoma, and for chromophobe renal cell carcinoma and oncocytoma, mirroring the tumor cell of origin within the nephron, respectively proximal tubule and distal tubule. This pattern was also described in multiplatform integrative analysis, suggesting that each tumor expression pattern might reflect the cell of origin as well as additional genetic and epigenetic alterations [3, 4]. TCGA cases were also used as a validation independent cohort, portraying similar results for clear cell, papillary and chromophobe renal cell carcinoma (oncocytomas are not represented in TCGA).

SMYD2, *SETD3*, *NO66* and *SETDB2* expression level were associated with prognosis in renal cell carcinoma in multivariable analysis, each one of them providing additional information to tumor stage. This might reflect histone alteration role not as driver event, but as modulator of tumor aggressiveness. In fact, our data also suggest that *RIOX2* might be involved in metastasis development. Additionally, mutations in histone modifying enzymes [1, 44], as well as some histone marks [10], as acetylation [45] and methylation (H3K4 [44], H3K27 [46], H3K9 [47], H3K36 [48]) have been associated with renal cell carcinoma prognosis. Altered expression of histone modifying enzymes, also observed in other studies [49, 50], might be another layer of histone deregulation with relevance for renal cell carcinoma progression.

Despite being promising prognostic biomarkers for renal cell carcinoma, validated in two large independent series, it would be valuable to further explore the biological function of *SMYD2*, *SETD3*, *NO66* and *SETDB2*, as well as performing additional

assessment in specific populations and clinical contexts. Moreover, renal cell carcinoma tumor heterogeneity [51, 52] should also be taken into account, especially when only limited samples are available.

6.2. GENERAL CONCLUSIONS AND PERSPECTIVES

Epigenetic alterations constitute promising biomarkers for clinical management of renal cell tumors, paralleling its involvement in renal tumorigenesis. These alterations, mostly promoter methylation and miRNA expression, might be useful as diagnostic biomarkers. Histone modifying enzymes might constitute useful prognostic markers in renal cell carcinoma.

The promising diagnostic and prognostic biomarkers here described, despite the validation performed, require additional assessment in large multicenter trials to support clinical implementation

For the diagnostic biomarkers, assessment of their clinical value as ancillary tool in renal mass biopsy specimens and/or formalin-fixed paraffin-embedded specimens would be valuable, as it could aid in rendering a more accurate diagnosis, especially if the diagnostic performance remained robust in small specimens, as well as in liquid biopsies.

It would also be relevant to assess the prognostic performance of these histone modifying enzymes in the context of targeted therapy in clinical series of patients with

metastatic renal cell carcinoma, as well as their performance as biomarkers predictive of response to therapy.

Moreover, the functional role of these histone modifying enzymes should be evaluated in renal cell carcinoma, to assess their potential as epigenetic drug targets or agents.

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APPENDIXES

OTHER RELEVANT CONTRIBUTIONS

The original research paper in this section was published in an international peer reviewed journal.

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MicroRNA profile: a promising ancillary tool for accurate renal cell tumour diagnosis

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Background: Renal cell tumours (RCTs) are clinically, morphologically and genetically heterogeneous. Accurate identification of renal cell carcinomas (RCCs) and its discrimination from normal tissue and benign tumours is mandatory. We, thus, aimed to define a panel of microRNAs that might aid in the diagnostic workup of RCTs.

Methods: Fresh-frozen tissues from 120 RCTs (clear-cell RCC, papillary RCC, chromophobe RCC (chRCC) and oncocytomas: 30 cases each), 10 normal renal tissues and 60 cases of *ex-vivo* fine-needle aspiration biopsies from RCTs (15 of each subtype validation set) were collected. Expression levels of miR-21, miR-141, miR-155, miR-183 and miR-200b were assessed by quantitative reverse transcription–PCR. Receiver operator characteristic curves were constructed and the areas under the curve were calculated to assess diagnostic performance. Disease-specific survival curves and a Cox regression model comprising all significant variables were computed.

Results: Renal cell tumours displayed significantly lower expression levels of miR-21, miR-141 and miR-200b compared with that of normal tissues, and expression levels of all miRs differed significantly between malignant and benign RCTs. Expression analysis of miR-141 or miR-200b accurately distinguished RCTs from normal renal tissues, oncocytoma from RCC and chRCC from oncocytoma. The diagnostic performance was confirmed in the validation set. Interestingly, miR-21, miR-141 and miR-155 expression levels showed prognostic significance in a univariate analysis.

Conclusion: The miR-141 or miR-200b panel accurately distinguishes RCC from normal kidney and oncocytoma in tissue samples, discriminating from normal kidney and oncocytoma, whereas miR-21, miR-141 and miR-155 convey prognostic information. This approach is feasible in fine-needle aspiration biopsies and might provide an ancillary tool for routine diagnosis.

Renal cell tumours (RCTs) account for ~4% of all adult neoplasms and 90–95% of all tumours arising in the kidney, ranking 14th in incidence worldwide, with an age-standardised mortality rate of 1.6 out of 100 000 (Ferlay *et al*, 2010). Renal cell tumours are morphologically and genetically heterogeneous (Baldewijns *et al*, 2008), comprising both malignant tumours (which are subclassified mainly as clear-cell renal cell carcinoma (ccRCC, 70–80% of cases), papillary RCC (pRCC, 10–15% of cases) and chromophobe RCC (chRCC, 5–10% of cases) and benign tumours (among which oncocytoma is the most common subtype; Kovacs *et al*, 1997).

Because histological subtypes differ in clinical aggressiveness and prognosis (Amin *et al*, 2002; Ficarra *et al*, 2006), accurate

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classification is required for appropriate patient management. Moreover, most RCTs are clinically silent at their earliest stages, and 20–30% are diagnosed when metastatic spread has already occurred (Abrahams *et al*, 2004). Although widespread use of imaging techniques (mainly ultrasonography) has increased detection of suspicious renal masses, prompting new pre-operative diagnostic challenges as histological diagnosis using needle biopsy material meets with important limitations, hampering an accurate categorisation in many instances (Shen *et al*, 2012). In this setting, diagnosis relies mainly on morphologic features, which show some overlap among tumour subtypes. The discrimination between chRCC (mainly the eosinophilic variant) and oncocytoma is one of the most critical and, sometimes, difficult differential diagnosis. Although these tumour types share some morphologic characteristics, chRCC is a malignant neoplasm, capable of local invasion and metastatic spread, whereas oncocytoma is a benign tumour just requiring a more conservative management.

Over the years, several attempts have been made to assist morphology in differential diagnosis between chRCC from oncocytoma, including immunohistochemical profiles (Abrahams *et al*, 2004; Lin *et al*, 2006; Shen *et al*, 2012), histochemical stains (Skinnider and Jones, 1999) and gene expression analysis (Lee *et al*, 2011). However, sensitivity and specificity of those techniques are suboptimal and prompt the need for more accurate biomarkers. Interestingly, some recent studies have attempted to discriminate among RCC subtypes using microRNA (miRNA) expression analysis. Nevertheless, most of those studies have mainly dealt with ccRCC or, when the most relevant histological subtypes were included, only a limited number of samples of each subtype have been analysed, precluding a definitive conclusion about their accuracy (Nakada *et al*, 2008; Petillo *et al*, 2009; Jung *et al*, 2009; Fridman *et al*, 2010; Juan *et al*, 2010; Youssef *et al*, 2011; Zhao *et al*, 2013). MicroRNAs are small non-coding RNAs (~22 nucleotides in length), which are involved in several essential biological processes such as cell differentiation, growth, apoptosis and proliferation (Esteller, 2011), and their deregulation has been implicated in tumorigenesis, including that of the kidney (Lu *et al*, 2005; Jung *et al*, 2009; Chow *et al*, 2010; White and Yousef, 2010). In addition to the differential expression patterns of miRNAs among RCT subtypes (Petillo *et al*, 2009; Jung *et al*, 2009; Juan *et al*, 2010; Valera *et al*, 2011; Youssef *et al*, 2011), altered miRNA expression might also provide relevant prognostic information (Neal *et al*, 2010).

In two recent reviews (Henrique *et al*, 2012; Jeronimo and Henrique, 2011), we found that five miRNAs (miR-21, miR-141, miR-155, miR-183 and miR-200b) had been reported as displaying diagnostic or prognostic value in RCT (Nakada *et al*, 2008; Petillo *et al*, 2009; Jung *et al*, 2009; Juan *et al*, 2010; Youssef *et al*, 2011). Thus, we aimed to confirm and extend those findings through expression analysis of a five miRNA panel in a single series of RCT, comprising the four major subtypes. We first assessed the expression levels of selected miRNAs in fresh-frozen tissues, focusing on the discrimination between benign and malignant tumours, as well as between chRCC and oncocytoma. In addition, the prognostic value of each miRNAs was determined. Finally, we validated our findings in a series of *ex-vivo* fine-needle aspiration biopsies from RCT to assess the feasibility of this approach as an ancillary tool in routine pathology.

MATERIALS AND METHODS

Clinical samples. A total of 130 fresh-frozen tissues were prospectively collected and included in this study, comprising 120 RCTs (30 cases of each of the four major subtypes (ccRCC, pRCC, chRCC and oncocytoma)) and 10 morphologically normal renal tissues (obtained from morphologic normal kidney tissue of patients subjected to nephrectomy due to upper urinary urothelial carcinoma; Table 1). In addition, a validation set comprising 60 *ex-vivo* fine-needle aspiration biopsies from RCT (15 of each subtype) was included. Samples from RCT patients were procured from patients diagnosed and treated at the Portuguese Oncology Institute – Porto (Portugal), between 2003 and 2007, who underwent partial or total nephrectomy, after obtaining informed consent. For each subtype, cases were consecutively selected until it reached 30 (for tissue samples) or 15 (for *ex-vivo* fine-needle aspiration biopsies) cases. This strategy was used to maximise the representation of the less common RCT types, thus ensuring that tumour heterogeneity in each subtype would be considered in the molecular analyses. Tumour tissue samples, obtained immediately after surgery, were snap-frozen, stored at −80 °C and subsequently cut in a cryostat for RNA extraction. *Ex-vivo* fine-needle aspiration biopsies of RCTs were obtained through 4–6 passes of a 23-gauge needle attached to a 10-ml syringe, then washed in PBS and stored at −80 °C until further use.

Routine histopathological assessment of all surgical specimens, in formalin-fixed paraffin-embedded tissue, was performed by an expert uropathologist (RH) and included tumour classification (WHO), grading (Fuhrman) and staging (TNM; Eble *et al*, 2004). Relevant clinical data were collected from clinical charts. This study, as well as the use of samples and access to clinical data, was approved by the Institutional Review Board (Comissão de Ética para a Saúde) of the Portuguese Oncology Institute – Porto.

Table 1. Clinical and pathological features of patients included in this study, including the data for the two sets of samples (fresh-frozen tissues and *ex-vivo* biopsies)

	Fresh-frozen tissues		*Ex-vivo* aspiration biopsies
	Tumour	Normal	Tumour
Number of patients, *n*	120	10	60
Age, median (range)	62 (30–84)	65 (20–83)	60 (30–82)
Gender, *n* (%)			
Male	71 (59.2)	7 (70.0)	35 (58.3)
Female	49 (40.8)	3 (30.0)	25 (41.7)
Histological subtype, *n* (%)		NA	
Clear-cell RCC	30 (25.0)		15 (25.0)
Papillary RCC	30 (25.0)		15 (25.0)
Chromophobe RCC	30 (25.0)		15 (25.0)
Oncocytoma	30 (25.0)		15 (25.0)
Pathological stage, *n* (%)		NA	
pT1	46 (38.3)		25 (41.7)
pT2	19 (15.9)		8 (13.3)
pT3	24 (20.0)		12 (20.0)
pT4	1 (0.8)		—
NA (oncocytoma)	30 (25.0)		15 (25.0)
Furhman grade, *n* (%)		NA	
1	3 (2.5)		0 (0.0)
2	27 (22.5)		12 (20.0)
3	44 (36.7)		20 (33.3)
4	16 (13.3)		12 (20.0)
NA	30 (25.0)		16 (26.7)

Abbreviations: NA = not applicable; RCC = renal cell carcinoma.

RNA extraction. Total RNA was extracted from fresh-frozen tissues and *ex-vivo* aspiration biopsies using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. Briefly, 1500 μl of Trizol reagent was added to each 2 ml tube and samples were homogenised using a rotor shaker. Tubes were incubated for 5–10 min at room temperature and then 300 μl of chloroform (Merck, Darmstadt, Germany) were added. Regarding biopsies the protocol was similar, but the Trizol reagent and chloroform volumes were 500 and 200 μl, respectively. Tubes were vigorously hand-shaked for 15 s and incubated for 3 min at room temperature, followed by a 15-min centrifugation at 12 000 g at 4 °C. Next, the upper phase was collected. RNA was purified using the PureLink RNA Mini Kit (Invitrogen), according to manufacturer's indications. RNA concentration and purity ratios were then evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). In addition, RNA quality was checked by electrophoresis in a 2% agarose gel.

Reverse transcription. Reverse transcription (RT) was performed using TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT human pool A (Applied Biosystems, Foster City, CA, USA). The reaction mixture had a final volume of 12 μl and included the following: 3 μl of total RNA (750 ng), 1.6 μl of megaplex RT primers (10 ×), 0.4 μl of dNTPs with dTTP (100 mM), 3 μl of MultiScribe reverse transcriptase (50 U μl^{-1}), 1.6 μl of 10 × RT buffer, 0.2 μl of RNase inhibitor (20 U μl^{-1}) and 0.4 μl of nuclease-free water. Reactions were performed in PCR tubes according to the following conditions: 40 cycles at 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 s, with a final incubation at 85 °C for 5 min.

Quantitative real-time RT–PCR. Quantitative RT–PCR (qRT–PCR) was performed using TaqMan Small RNA Assays (Applied Biosystems) in a 7500 Real-Time PCR system (Applied Biosystems), according to the recommended protocol. Briefly, for each reaction 0.5 μl of TaqMan Small RNA Assay (20 ×), 0.75 μl of RT product, 5 μl of TaqMan Universal PCR Master Mix II no UNG (2 ×) and 3.75 μl of nuclease-free water were added. According to the manufacturer's instructions, the protocol conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Expression levels of the five selected miRNAs (hsa-miR-21: Tm000397; hsa-miR-141: Tm000483; hsa-miR-155: Tm002626; hsa-miR-183: Tm002269; and hsa-miR-200b: Tm002251) were assessed in triplicate for each sample and two water blanks were added to each plate as negative controls.

Results from the qRT–PCR were analysed using the 7500 Software version 2.0.5 (Applied Biosystems). Levels of miRNA expression were determined using the relative standard curve method (Biosystems, 2004). In each sample, the mean quantity of each miRNA was normalised to the mean quantity for the endogenous controls RNU48 and RNU6B, according to the following formula: miRNA expression = candidate miRNA expression mean quantity/((RNU48 mean quantity + RNU6B mean quantity)/2). Results were then multiplied by 10 000 for easier tabulation.

Statistical analysis. Differences in expression levels of the candidate miRNAs among the different histological subtypes were first analysed using a non-parametric Kruskal–Wallis test, followed by pairwise comparisons using non-parametric Mann–Whitney *U*-test, when appropriate. The relationship between miRNA expression and clinicopathological variables (gender, Fuhrman grade (recoded into two groups: grades 1–2 *vs* 3–4) and pathological stage (recoded into two groups: pT1–pT2 *vs* pT3–pT4)) was evaluated using Mann–Whitney *U*-test. Spearman's non-parametric correlation tests were additionally carried out to ascertain correlations between age and miRNA expression levels. Receiver operator characteristic (ROC) curves were constructed by plotting the true-positive rate (sensitivity) against the false-positive rate (1 − specificity) for each miRNA and for the best combination of miRNAs. The selection of the best miRNA panel was achieved using logistic regression, and the areas under the curve (AUCs) were calculated to assess the panel's diagnostic performance. Disease-specific survival (DSS) curves (Kaplan–Meier with log-rank test) were computed for clinical variables (age, gender, histological subtype, Fuhrman grade and pathological stage) and miRNA expression levels. A Cox regression model comprising all significant variables (multivariate test) was computed to assess the relative contribution of each variable to the follow-up status. For the purpose of survival analyses, all cases were coded based on each miRNA expression levels, using the median value as the cut-off value. Statistical analysis was performed using SPSS for Windows, version 20.0 (SPSS, Chicago, IL, USA) and differences were considered statistically significant when $P<0.05$. For multiple comparisons, the *P*-value was adjusted according to Bonferroni's method (i.e., the level of significance was adjusted to $P<0.05/n$, in which n represents the number of groups under comparison).

RESULTS

MicroRNA expression levels and clinicopathological correlates. The relative expression levels of miR-21, miR-141, miR-155, miR-183 and miR-200b were determined in fresh-frozen tissues of 120 RCTs and 10 normal renal tissue samples. Relevant clinical and histopathological data are displayed in Table 1. No significant differences in age or gender between RCTs patients and normal tissue donors were apparent. No statistically significant associations were disclosed between miR expression levels and any of the clinicopathological features (age, gender, Fuhrman grade categories or pathological stage). Renal cell tumours showed significantly lower expression levels of miR-21, miR-141 and miR-200b compared with that of normal tissues ($P<0.001$ for all; Figure 1A and Supplementary Table 1). Moreover, expression

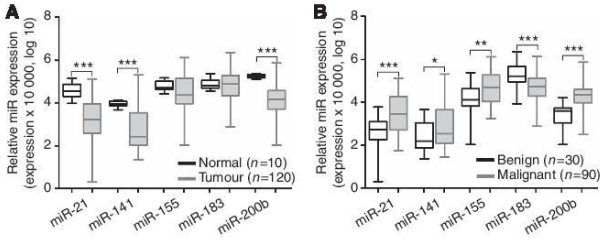

Figure 1. Distribution of miRNA expression levels in kidney tissues. (**A**) Normal *vs* tumour tissues. (**B**) Benign *vs* malignant tumour tissues. Statistically significant differences are represented as ***$P<0.001$, **$P<0.01$ and *$P<0.02$.

levels of all candidate miRNAs differed significantly between benign and malignant RCTs. Oncocytomas displayed lower expression levels for all tested miRs, except miR-183 (Figure 1B and Supplementary Table 2).

Although there was a wide expression range within the four RCT subtypes, with a significant degree of overlap, expression levels of all miRs differed significantly among them ($P<0.001$ for all, Kruskal–Wallis test; Table 2). Pairwise comparisons are shown in Table 3 and graphically illustrated in Figure 2. In general, oncocytomas displayed the lower miR expression levels, significantly differing from pRCC or ccRCC regarding four miRs (miR-21, miR-155, miR-183 and miR-200b), and from chRCC in two miRs (miR-141 and miR-200b). Interestingly, ccRCC and pRCC only differed for miR-155 expression levels, whereas chRCC differed from ccRCC and pRCC for miR-21, miR-141 and miR-155 expression levels. In addition, miR-183 expression levels were also different between chRCC and ccRCC (Table 3). Thus, reduced expression of miR-200b surfaced as the most discriminative feature between oncocytomas and RCCs.

Diagnostic performance of miRNA expression levels in tissue samples. Performance of the five studied miRs was assessed in three different settings: identification of RCTs (*vs* normal renal tissue), discrimination of malignant from benign tumours and distinction of chRCC from oncocytoma. For that purpose, the cut-off value corresponded to the best performance of each miRNA according to the respective ROC curve analysis. Validity and information estimates for each marker and for the best combination of markers are displayed in Table 4. Receiver operator characteristic curve analysis showed that a panel comprising expressions of miR-141 and miR-200b allowed for the discrimination between RCT and normal renal tissue with 99.2% sensitivity and 100% specificity, corresponding to an AUC of 0.991. In addition, the same panel allowed for the differentiation between benign and malignant tumours with 85.6% sensitivity and 100% specificity, displaying an AUC of 0.912. Furthermore, expression levels of miR-141 and miR-200b also distinguished chRCC from oncocytoma with 90% sensitivity and 100% specificity, corresponding to an AUC of 0.90 (Figure 3A and B).

Survival analysis. The median follow-up of this series of RCT patients was 65 months (range: 1–120 months). A total of 12 patients (13.3%) have died from RCC during this period. Disease-specific survival analysis showed that tumour subtype ccRCC or pRCC and higher pathological tumour stage (pT3–T4) were significantly associated with worse outcome ($P=0.011$ and $P<0.001$, respectively; Figure 4A and B). Although age at diagnosis over 62 years was associated with worse DSS ($P=0.035$), gender and Fuhrman grade did not disclose any prognostic value within the available follow-up time. Concerning miRNA expression levels, miR-200b and miR-183 did not exhibit any prognostic value. However, higher expression levels of miR-21 and miR-155, and lower expression levels of miR-141 were associated with worse DSS ($P=0.006$, $P=0.037$ and $P=0.024$, respectively; Figure 4C–E). However, in multivariate analysis only pathological stage independently predicted prognosis, whereas miRNA expression levels did not retain an independent prognostic value (Supplementary Table 3).

Validation of the miRNA panel in *ex-vivo* aspiration biopsies. The two best-performing miRNA in tissue samples, miR-141 and miR-200b, were then selected for analysis in *ex-vivo* samples. This set comprised 60 *ex-vivo* fine-needle aspiration biopsies. Relevant clinical and histopathological data are summarised in Table 1 and the relative expression levels for each miR are depicted in Supplementary Table 4 and Supplementary Figures S1 and S2.

Remarkably, expression levels of this panel of miRNAs were able not only to distinguish benign from malignant RCT with 73.3% sensitivity and a 100% specificity (AUC of 90.4%), but also oncocytoma from chRCC with 100% sensitivity and 100% specificity (AUC of 100%; Figure 3C and D; Table 5).

DISCUSSION

In this study, we aimed to define a small set of miRs that might allow for accurate identification of RCTs, as well as for discrimination between oncocytoma and RCCs, especially chRCC. This would be of clinical relevance, as diagnostic workup of suspicious renal masses incidentally found by abdominal ultrasonography is increasingly more frequent and demanding. Indeed, each RCT subtype displays quite dissimilar clinical behaviour, ranging from totally benign to overtly malignant, and successful

Table 3. Comparison of microRNA expression among renal cell tumour subtypes in fresh-frozen tissues

	P-value[a], M–W test				
	miR-21	miR-141	miR-155	miR-183	miR-200b
Oncotytoma *vs* ccRCC	<0.001	NS	<0.001	<0.001	<0.001
Oncocytoma *vs* pRCC	<0.001	NS	0.012	<0.001	<0.001
Oncotytoma *vs* chRCC	NS	0.001	NS	NS	0.001
pRCC *vs* ccRCC	NS	NS	0.003	NS	NS
ccRCC *vs* chRCC	<0.001	0.002	<0.001	<0.001	NS
pRCC *vs* chRCC	<0.001	<0.001	0.002	NS	NS

Abbreviations: ccRCC = clear-cell renal cell carcinoma; chRCC = chromophobe RCC; M–W = Mann–Whitney test; NS = not significant; pRCC = papillary RCC.
[a]Statistically significant when $P<0.0125$, Bonferroni's correction.

Table 2. Distribution of microRNA expression levels among different histological subtypes in fresh-frozen tissues

	Oncocytoma	chRCC	pRCC	ccRCC	*P*-value, K–W
miR-21	5.3 (0.02–60.9)	4.0 (0.8–560.2)	47.9 (0.6–689.3)	155.5 (3.5–1325.8)	<0.001
miR-141	7.9 (0.2–45.9)	83.5 (0.3–552.2)	76.8 (0.3–2063.3)	25.75.7 (0.3–301.2)	<0.001
miR-155	374.9 (1.1–233.7)	339.6 (14.5–5340.1)	1054 (17.1–4595.9)	3148.8 (23.74–13299)	<0.001
miR-183	5034.7 (87.1–23207.1)	1690.3 (18.8–8013.8)	1350.1 (7.7–13865)	512.5 (15.9–2360.7)	<0.001
miR-200b	40.3 (1.1–161)	367.9 (3.3–1244)	611.6 (4.8–7445.1)	249.1 (38.1–930.2)	<0.001

Abbreviations: ccRCC = clear-cell renal cell carcinoma; chRCC = chromophobe RCC; K–W = Kruskal–Wallis test; pRCC = papillary RCC.

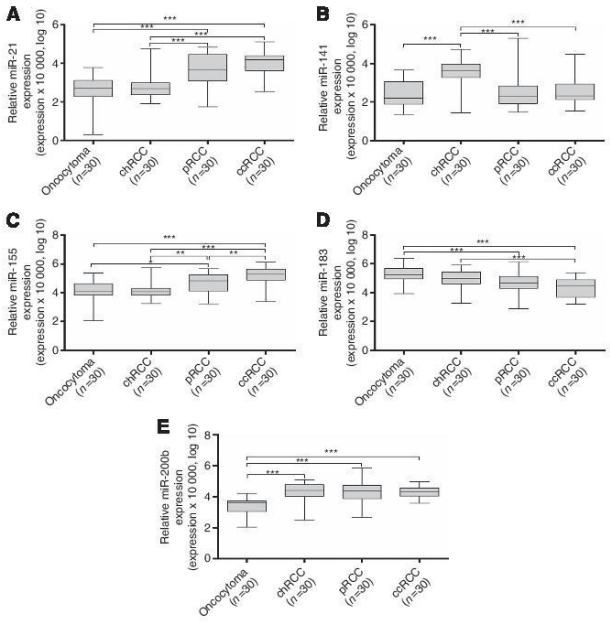

Figure 2. Distribution expression levels of (**A**) miR-21, (**B**) miR-141, (**C**) miR-155, (**D**) miR-183 and (**E**) miR-200b according with the histological subtype of RCTs. Statistically significant differences are represented as ***$P<0.001$, **$P<0.003$ and *$P<0.0125$.

Table 4. Validity estimates for each tested miR and for the best combination of miRs in each diagnostic setting, in fresh-frozen tissues

	miR-21	miR-141	miR-155	miR-183	miR-200b	miR-141 or miR-200b
RCT *vs* normal renal tissue						
SE	76.7	81.7	—	—	97.5	99.2
SP	100	100	—	—	100	100
PPV	100	100	—	—	100	100
NPV	26.0	31.0	—	—	77.0	90.9
Accuracy	78.0	83.0	—	—	98.0	99.2
AUC	89.9	89.7	—	—	98.7	99.1
RCC *vs* oncocytoma						
SE	48.9	25.6	50.0	72.2	96.7	85.6
SP	93.3	100	83.3	73.3	90.0	100
PPV	95.7	100	90.0	89.0	96.7	100
NPV	37.8	13.0	35.2	46.8	67.5	69.8
Accuracy	60.0	33.0	58.3	72.5	95.0	89.2
AUC	75.9	64.9	66.7	75.1	91.4	91.4
chRCC *vs* oncocytoma						
SE	—	76.7	—	—	83.3	90.0
SP	—	86.7	—	—	90.0	100
PPV	—	85.2	—	—	89.3	100
NPV	—	78.7	—	—	84.4	90.9
Accuracy	—	81.6	—	—	86.7	95.0
AUC	—	81.9		—	89.6	90.0

Abbreviations: AUC = area under the curve; chRCC = chromophobe RCC; NPV = negative predictive value; PPV = positive predictive value; RCC = renal cell carcinoma; RCT = renal cell tumour; Se = sensitivity; Sp = specificity

pretherapeutic cytological or histological assessment is limited (Amin *et al*, 2002; Ficarra *et al*, 2006). Only a few studies addressed the use of miRNA expression as biomarkers for RCTs detection, and these have been mainly restricted to the ccRCC subtype, or have only analysed a very limited number of samples (Jung *et al*, 2009; Petillo *et al*, 2009; Juan *et al*, 2010; Youssef *et al*, 2011; Redova *et al*, 2012; Zhao *et al*, 2013). After an extensive review of published literature, we selected five miRNAs (miR-21, miR-141, miR-155, miR-183 and miR-200b) with putative diagnostic and prognostic value (Jeronimo and Henrique, 2011; Henrique *et al*, 2012), and tested them in a relatively large set of tissue samples that comprised the major histological subtypes. To ascertain their clinical and pathological relevance, a validation study was subsequently performed in a set of *ex-vivo* fine-needle aspiration biopsies.

Of the five miRs tested, three (miR-21, miR-141 and miR-200b) were significantly downregulated in RCTs compared with normal renal tissue. In previous reports, miR-21 was found to be upregulated in RCT (Juan *et al*, 2010; Faragalla *et al*, 2012; Zaman *et al*, 2012), which apparently contradicts our results. However, in those studies, normal renal tissue was obtained from nephrectomy specimens harbouring RCT, which did not occur in our study. This is an important issue, as we have previously shown that morphologically normal renal tissue from kidneys harbouring RCT display epigenetic alterations in line with the respective tumours (Costa *et al*, 2007). Remarkably, variations in miR-21 expression among RCT subtypes observed in our study matches that reported by Faragalla *et al* (2012), with ccRCC depicting the highest median levels, followed by pRCC, chRCC and oncocytoma. Indeed, only miR-21 expression levels of 'normal renal tissue' are notably different between our results and their study

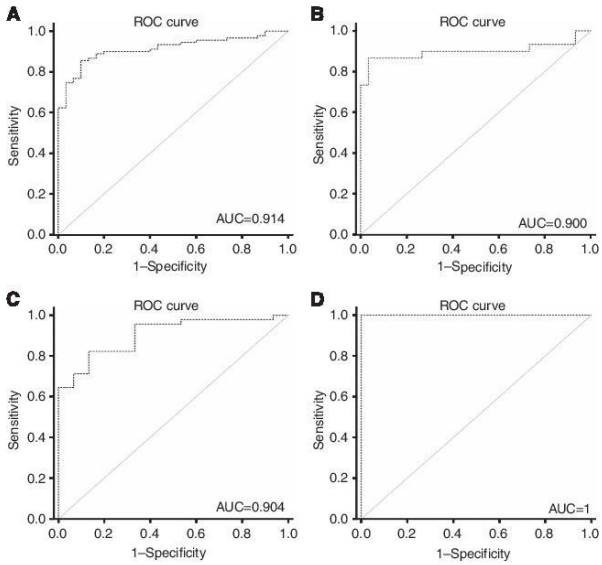

Figure 3. ROC curves. ROC curves evaluating the performance of the gene panel (miR-141 and miR-200b) as a biomarker for malignant renal tumours (A and C) and as a biomarker of chRCC (B and D). (**A** and **B**) Performed in kidney tissue samples; (**C** and **D**) performed in *ex-vivo* aspiration renal biopsies.

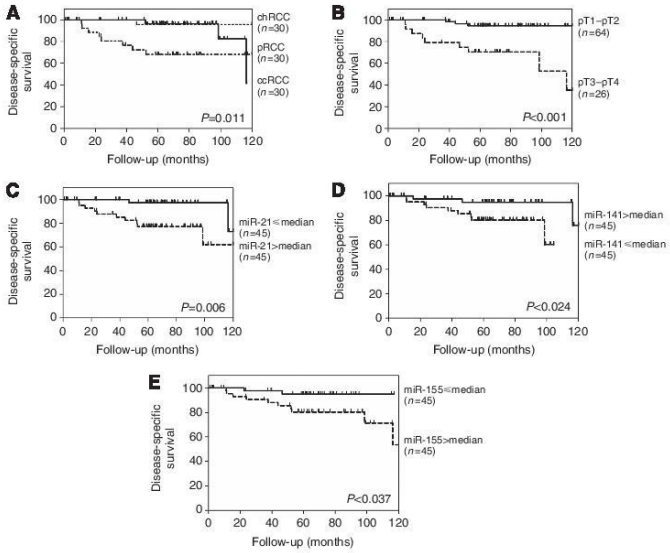

Figure 4. Disease-specific survival according to pathological and molecular parameters. (**A**) Histopathological classification; (**B**) pathological stage; (**C**–**E**) miR expression levels.

(Faragalla *et al*, 2012). These findings prompt the need for an adequate definition of 'normal tissue', as the interpretation of results in tumours might be considerably biased.

Concerning miR-141, our results corroborate those of two previous reports (Nakada *et al*, 2008; Fridman *et al*, 2010). Thus, higher miR-141 expression levels seem to be a hallmark of chRCC

Table 5. Validity estimates for each tested miR and for the best combination of miRs in each diagnostic setting, in *ex-vivo* aspiration biopsies

	(%)	miR-141	miR-200b	miR-141 or miR-200b
RCC *vs* oncocytoma				
	SE	35.5	73.3	73.3
	SP	93.3	93.3	100
	PPV	94.1	97.1	100
	NPV	34.5	53.8	55.0
	Accuracy	50.0	78.3	80.0
	AUC	57.5	88.4	90.4
chRCC *vs* oncocytoma				
	SE	73.3	100	100
	SP	93.3	100	100
	PPV	91.6	100	100
	NPV	77.7	100	100
	Accuracy	83.3	100	100
	AUC	84.4	100	100

Abbreviations: AUC = area under the curve; chRCC = chromophobe RCC; NPV = negative predictive value; PPV = positive predictive value; RCC = renal cell carcinoma; Se = sensitivity; Sp = specificity;

and might constitute a valuable biomarker for discrimination from oncocytoma. Strikingly, a miRNA profiling of ccRCC also identified miR-141 (and 200b) as being downregulated in ccRCC, although with concurrent upregulation of miR-155 (Jung *et al*, 2009). These results are in line with ours, as we found that the highest miR-155 expression levels in ccRCC and pRCC significantly differed from those of oncocytoma and chRCC. Our findings concerning miR-200b mirror those of Youssef *et al* (2011), although in a smaller data set. Interestingly, in our larger data set we were able to demonstrate that miR-200b expression levels were significantly lower in oncocytomas compared with all RCC subtypes. Overall, the comparisons of miR expression levels among RCT subtypes also denote the common origin (segment of the nephron) of ccRCC and pRCC on one hand, and of chRCC and oncocytoma on the other hand, emphasising the importance of searching for discriminative biomarkers, which might enable accurate identification of each RCT subtype.

Interestingly, a panel comprising miR-141 and miR-200b demonstrated the best performance in frozen-tissue samples, displaying AUC values ranging from 90.0 to 99.1. Although these results are interesting *per se*, its clinical usefulness depends on the possibility of using it in diagnostic samples. For that purpose, we further validated this biomarker panel in a set of fine-needle aspiration biopsies performed *ex vivo*. Although this procedure is not completely equivalent to an imaging-guided diagnostic fine-needle aspiration biopsy performed in a patient (which may yield lower amounts of tumour cells), it is, nonetheless, the best approximation without jeopardising patients' diagnosis. On the other hand, because the nephrectomy specimen is already available its histopathological characterisation is guaranteed, whereas diagnostic biopsies may not be followed by surgical excision, thus precluding accurate tumour classification for comparison purposes. Remarkably, the biomarker panel performance in *ex-vivo* biopsies was comparable to that of fresh-frozen tissues. To the best of our knowledge, this is the first attempt to demonstrate the feasibility of using miRs as tumour biomarkers in renal tumour biopsies, and may thus constitute a significant step forward in the development of epigenetic-based biomarkers for management of RCC suspects.

The clinical significance of our findings could be extended if miRNA expression levels might convey prognostic information. Thus, we performed DSS analysis using expression levels determined in fresh-frozen-tissue samples. As expected, tumour subtype and pathological stage were of prognostic value in univariate analysis, although only the later showed independent prognostic value in multivariate analysis. Remarkably, miR-21, miR-141 and miR-155 expression levels also displayed prognostic significance in RCC, although only in univariate analysis. A possible explanation for these findings may lie in the association between specific miR expression levels and tumour subtypes. Indeed, whereas for miR-21 and miR-155 the association with poorer DSS was observed for higher (> median) expression levels, the opposite was verified for miR-141. Interestingly, higher miR-21 and miR-155 expression levels and lower miR-141 expression levels were associated with pRCC and ccRCC subtypes, which displayed the worse prognosis compared with that of chRCC. The fact that tumour subtype did not surfaced as independent prognostic parameter for DSS in multivariate analysis is most likely due to the association between tumour subtype and pathological stage, as pT3–4 tumours were mostly of pRCC or ccRCC subtype. Our findings concerning miR-21 and miR-141 are corroborated by previous reports, although with generally smaller patient cohorts (Jung *et al*, 2009; Faragalla *et al*, 2012; Zaman *et al*, 2012). In addition, the prognostic value of miR-155 expression levels has been reported for breast cancer (Song *et al*, 2012) and non-small cell lung cancer (Yanaihara *et al*, 2006; Yang *et al*, 2013), whereas miR-21 and miR-141 expression seem to be of prognostic significance in non-small cell lung cancer (Yanaihara *et al*, 2006; Yang *et al*, 2013) and colon cancer (Cheng *et al*, 2011), respectively.

The aforementioned association of specific miRs altered expression and RCT subtype might also provide clues concerning the cause of miR dysregulation. Renal cell tumour subtypes display characteristic chromosomal aberrations, including whole or partial deletions and duplications (Baldewijns *et al*, 2008). Strikingly, some of those alterations might explain the altered pattern of miR expression. For instance, miR-200b is mapped at 1p36.33 and loss of 1p or of the whole chromosome 1 is frequently observed in oncocytoma and chRCC. On the other hand, miR-21 and miR-155 are mapped at 17q23.1 and 21q21.2–21.3, which are frequently lost chromosomal regions in chRCC. Conversely, pRCC, which commonly show gain of chromosome 17, are among the RCT subtypes with higher miR-21 expression levels. However, other variations in miR expression might not be explained by chromosomal-level alterations and the respective cause(s) remain to be investigated.

CONCLUSIONS

Herein we demonstrate that expression levels of a panel of two miRNAs (miR-141 or miR-200b) allows for accurate distinction of normal kidney from RCT tissue samples, as well as for accurate discrimination among RCT subtypes, including the separation of benign from malignant RCT. Furthermore, the selected miR panel is able to convey prognostic information, although not independent of tumour subtype or pathological stage. Importantly, the same panel displays an impressive performance for accurate detection of RCC in clinical samples obtained from *ex-vivo* fine-needle aspiration biopsies, demonstrating the feasibility of this approach in routine diagnostic practice.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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